Molecular studies on the fish pathogen *Yersinia ruckeri*

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ABSTRACT

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Yersinia ruckeri is the aetiological agent of enteric redmouth (ERM), a disease of salmonids, notably rainbow trout (*Oncorhynchus mykiss*, Walbaum). Until the 1990s, prophylaxis was achieved using a formalin-inactivated whole-cell vaccine of a motile (= flagellin producing) *Y. ruckeri* strain. However, outbreaks of ERM have since occurred in vaccinated livestock which heralded the emergence of a new biogroup. In addition to giving a negative result for the Voges–Proskauer (VP) reaction and the production of an extracellular lipase, strains responsible for the majority of these new outbreaks in vaccinated stock were non-motile and unable to produce detectable flagellin. It was the aim of this study to determine what protective role flagellin may have towards *Y. ruckeri* infection, both as a component of the whole-cell vaccine and as a vaccine in itself (i.e. sub-unit vaccine).

Results showed that protection against bacterial challenge, either with a motile or non-motile *Y. ruckeri* strain, was not entirely dependent on the presence of flagellin within the whole-cell vaccine. On the other hand, administering native flagellin (50 µg/fish) via intraperitoneal injection (without adjuvant) resulted in excellent levels of protection (relative percent survival = 100%) against challenge 28 days post-vaccination with a flagellin-producing (YR1) or flagellin-devoid (R1) *Y. ruckeri* strain. Use of recombinant flagellin (r-flagellin) as a vaccine again confirmed the protective properties against challenge with both YR1 and R1 strains, even at lower concentrations i.e. 10 µg/fish. Protection was also conferred after a relatively short period of time (14 days) without any detrimental effect on health or weight of the fish. Thus flagellin has the potential to be an efficacious, non-specific sub-unit vaccine for rainbow trout.

Analysis of whole cell proteins by SDS-PAGE from both motile and non-motile isolates demonstrated that highly virulent EX5 isolates which caused disease in vaccinated livestock were overexpressing a 30 to 40 kDa protein. 2D SDS-PAGE and Maldi-tof mass spectrometry identified this protein as outer membrane protein A (OmpA). However, attempts to disrupt the gene encoding the OmpA protein (*ompA*) using transposon mutagenesis and PCR screening failed to isolate a mutant with a transposon within the gene of interest (*ompA*::Tn-RL27).

To my mother,

Carol Scott

"Once you eliminate the impossible, whatever remains, no matter how improbable, must be the truth."[1]

***In* "Sherlock Holmes: A Scandal in Bohemia"[1]**
Sir Arthur Conan Doyle
Scottish physician, patriot and man of letters (1859-1930)

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DECLARATION

I, Callum J. W. Scott, hereby declare that I am the author of this thesis. All the work described in this thesis is my own, except where stated in the text. Results presented in this work have not been previously used in an application for a higher degree. All sources of information are acknowledged by means of references.

Callum J. W. Scott

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ABBREVIATIONS

%	percent
~	approximately
<	less than
>	greater than
+	plus or supplemented with
±	plus or minus, error margin
x	multiply/times
≤	less than or equal to
≥	greater than or equal to
°C	degree centigrade
‰	parts per thousand
APS	ammonium persulphate
BLAST	basic local alignment search tool
bp	base pair (DNA)
CFU	colony forming units
cm	centimetre
DIG	digoxigenin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECP	extracellular product
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
g	gram
g	gravitational force
h	hour
HRP	horse-radish peroxidase
i.e.	'id est': that is
IEF	isoelectric focusing
IgM	immunoglobulin M
IMAC	immobilized metal ion affinity chromatography
i.p.	intraperitoneally
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
kbp	kilo base pair
L	litre
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LD_{50}	lethal dose to kill 50% of the population
LD_{60}	lethal dose to kill 60% of the population
LPS	lipopolysaccharides
M	mole
MCA	monoclonal antibody
mg	milligram
min	minute
ml	millilitre
mM	millimolar
NA	nutrient agar
NB	nutrient broth

NCBI	national centre for biotechnology information
nm	nanometer (= unit of wavelength)
OD	optical density
OMP	outer membrane protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
PMSF	phenylmethanesulfonylfluoride
r-flagellin	recombinant flagellin
RNA	ribonucleic acid
RPS	relevant percent survival
SDS-PAGE	sodium-dodecyl sulphate polyacrylamide gel electrophoresis
spp.	species
SSC	saline-sodium citrate
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TSA	tryptone soya agar
TSB	tryptone soya broth
UV	ultraviolet
V	volt
v/v	volume to volume ratio
WT	wild-type
w/v	weight to volume ratio
w/w	weight to weight ratio
Y. ruckeri	*Yersinia ruckeri*
µg	microgram
µl	microliter
µm	micrometer

CHAPTER 1: INTRODUCTION

1.1 Aquaculture

The farming of aquatic organisms, including fish, crustaceans and molluscs, often known as aquaculture or aquafarming, is one of the fastest growing animal-food-producing industries in the world. Aquaculture currently provides nearly half (approx. 46 %) of all fish consumed by the global population and in 2008 alone was thought to have produced 148 million tonnes of fish (Food and Agricultural Organisation of the United Nations [FAO], (2010). This number includes 115 million tonnes of fish consumed by humans. As of 2010, the human population stood at approximately 6.29 billion people and is estimated to rise to 10.1 billion by the year 2100 (United Nations. Dept. of Economic and Social Affairs. Population Division., 2011). This will place aquaculture under considerable pressure to produce the amount of aquatic food required to meet the demands of an increasing human population. Fish must therefore be able to grow quickly under potentially adverse environmental conditions (i.e. varying temperatures and water quality) with minimal costs to the aquaculturist, yet still produce a final product which is palatable and safe for consumption. An important example is rainbow trout (*Oncorhynchus mykiss*, Walbaum), which has been successfully reared in captivity for over 100 years.

1.2 Rainbow trout

Rainbow trout is native to North America and since 1874 has been introduced to all continents (with the exception of Antarctica) for aquaculture and recreational angling (FAO, 2010). Since the commercialisation of a fish pellet diet in the 1950s, production of this species has increased considerably (Fig 1.1), particularly in Europe, North America, Japan, Chile, and Australia. In Scotland, an estimated 7,600 tonnes of rainbow trout were produced in 2008 alone (Marine Science Scotland, 2008). This fish species can be grown quickly over a range of temperatures and does not require stringent water quality. Moreover, a great deal of knowledge has been generated for rainbow trout regarding reproduction, genetics, immunology, nutrition and health. Unfortunately, with the rapid expansion of fish farming, the numbers of disease outbreaks have increased considerably in recent years.

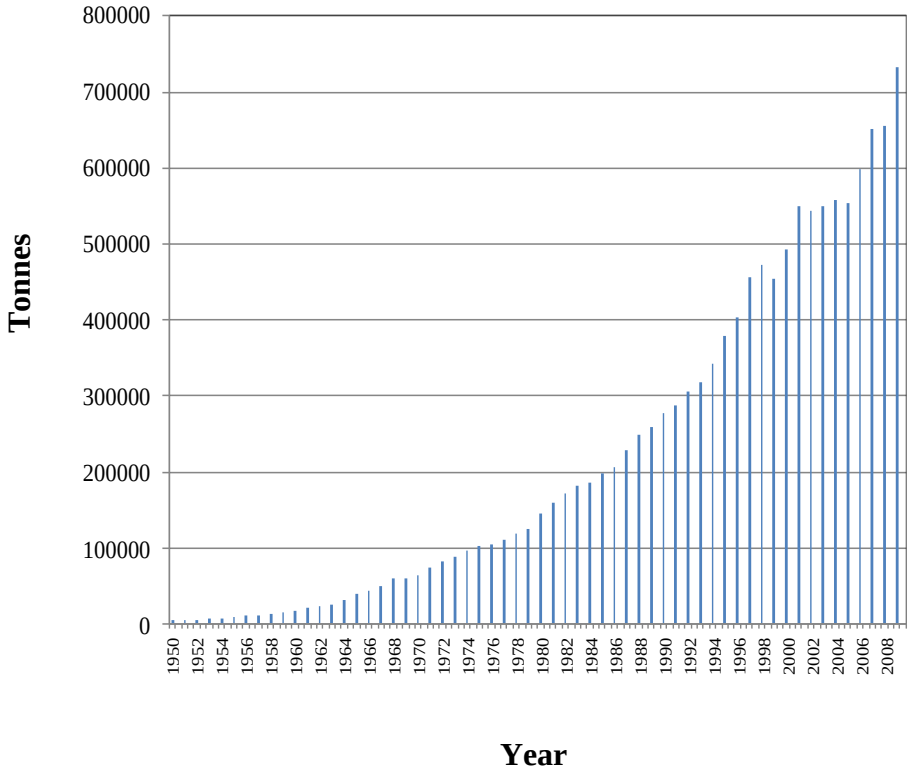


Figure 1.1: Global production of rainbow trout (in tonnes) by aquaculture between the years 1950 and 2009. Data obtained from the Food and Agriculture Organization of the United Nations (2010).

1.3 Diseases of rainbow trout

Outbreaks of disease can cause significant morbidity and mortality, often resulting in severe financial losses for the industry. A number of fish pathogens, both bacterial and viral, can cause disease in rainbow trout (some topical pathogens are shown in Table 1.1) and have since become the basis of further research (for extensive reviews see Gomez-Casado *et al*., 2011, Walker and Winton, 2010, Austin and Austin, 2007, Roberts, 2001, Toranzo *et al*., 2005).

Table 1.1: Fish pathogens currently problematic for aquaculture (after Roberts, 2001, Austin and Austin, 2007).

Pathogen	***Disease***
Bacterial pathogens:	
Gram-positive:	
Bacillus spp.	Septicaemia, bacillary necrosis
Lactococcus garvieae (formerly *Enterococcus seriolicida*)	Streptococcosis
Mycobacterium marinum	Mycobacteriosis (form of fish tuberculosis)
Renibacterium salmoninarum	Bacterial kidney disease (BKD)
Streptococcus iniae (formely *S. shiloi*)	Streptococcosis
Streptococcus parauberis	-
Gram-negative:	
Aeromonas bestiarum	Ulceration
Aeromonas hydrophila (formerly *A. liquefaciens* and *A. punctata*)	Haemorrhagic septicaemia, red sore disease and fin rot
Aeromonas salmonicida	Furunculosis, ulcer disease and carp erythrodermatitis
Edwardsiella ictaluri	Enteric septicaemia of catfish
Edwardsiella tarda	Edwardsiellosis and redpest
Flavobacterium psychrophilum (formerly *Cytophaga psychrophila*)	Cold water disease, necrotic myositis and rainbow trout fry syndrome
Hafnia alvei	Haemorrhagic septicaemia
Pasteurella skyensis	Pasteurellosis
Photobacterium damselae subsp. *piscicida* (formerly *Pasteurella piscicida*)	Pseudotuberculosis
Piscirickettsia salmonis	Coho salmon syndrome and salmonid rickettsial syndrome
Pseudomonas anguilliseptica	Red spot and winter disease
Salmonella enterica subs. *arizonae* (= *Sal. choleraesuis* subsp. *arizonae* and *Sal. arizonae*)	Septicaemia
Serratia liquefaciens	-
Serratia marcescens	-
Tenacibaculum maritimum (formerly *Flexibacter maritimus*)	Bacterial stomatitis, gill disease and necrotic myositis
Vibrio anguillarum	Vibriosis
Vibrio cholerae (non-O1)	Septicaemia

Vibrio harveyi (formerly *V. carchariae* and *V. trachuri*)	Eye disease, necrotising enteritis and vasculitis
Vibrio ordalii	Vibriosis
Vibrio (= Aliivibrio) salmonicida	Vibriosis (coldwater) and Hitra disease
Vibrio vulnificus	Septicaemia
Yersinia ruckeri	Enteric redmouth (ERM)
Viral pathogens:	
Infectious hematopoietic necrosis virus (IHNV)	General viraemia
Infectious pancreatic necrosis virus (IPNV)	-
Infectious salmon anaemia virus (ISAV)	-
Salmon alphaviruses (SAVs)	-
Spring viraemia of carp virus (SVCV)	-
Viral haemorrhagic septicaemia virus (VHSV)	-

The establishment of disease is often multi-factorial insofar as it depends upon the environment, the health status of the fish, and the virulence/infective dose of the pathogen. Many diseases are associated with so called 'opportunistic pathogens' which maintain an endemic (= carrier) state within the environment or host, only causing disease following another disease process (i.e. secondary infection) or stressful event (Austin and Austin, 2007). Nonetheless, there are some highly virulent bacterial pathogens, notably *A. salmonicida* and *V. anguillarum*, which do not require the host to be immunocompromised (Roberts, 2001). Resurgences in certain diseases initially controlled with some success by vaccination, such as enteric redmouth disease (ERM), are becoming increasingly problematic for aquaculture.

1.4 Enteric redmouth disease (ERM)

ERM, sometimes known as yersiniosis, "Hagerman redmouth" or "salmonid blood spot", is a bacterial septicaemia which can affect farmed salmonids, particularly rainbow trout. In severe cases, ERM outbreaks can result in the loss of 30-70% of

fish stocks (Horne and Barnes, 1999). The aetiological agent is *Yersinia ruckeri*, a bacterium originally reported in the Hagerman valley of Idaho, USA, during the 1950s by Rucker *et al.* (1966). This organism has since been found within North and South America (Ross *et al*., 1966, Stevenson and Daly, 1982, Bravo and Kojagura, 2004), Australia (Bullock *et al*., 1978, Llewellyn, 1980), South Africa (Bragg and Henton, 1986), Iran (Soltani *et al.*, 1999), and throughout most parts of Europe (Davies and Frerichs, 1989, Roberts, 1983, Lesel *et al*., 1983, Fuhrmann *et al*., 1983, Bush, 1978, Meier, 1986, Romalde *et al*., 1994b).

1.4.1 Susceptibility to ERM

Even though the majority of ERM cases occur in rainbow trout, other fish species of commercial importance are susceptible to infection (Furones *et al*., 1993) (Table 1.2). Some reports have suggested that larger fish are more prone to ERM infections, although size alone cannot be a determining factor (Rucker, 1966). ERM epizootics in healthy, vaccinated livestock are extremely rare. When disease outbreaks do occur they commonly result in low-level mortalities, often leading to acute, large-scale epizootics if chronically infected fish become stressed (Hunter *et al*., 1980). Stressors include high stocking densities, extremes in temperatures, poor diets and over-handling. Pollutants, such as excess ammonia, are common stressors in aquaculture (Austin, 1998). Fish must also be adequately vaccinated against different forms of virulent *Y. ruckeri* strains to ensure protection. Incubation times for this disease in naturally infected fish range from 5 to 10 days at 13-15°C, albeit dependent upon the health status of the host (Bullock and Cipriano, 1990).

1.4.2 Clinical signs of ERM

Disease associated with ERM, as recently reviewed by Tobback *et al.* (2007), may be acute or chronic depending on the size of fish. Generally, fingerling fish show acute cases of disease, whereas the condition can become chronic in larger fish. In acute form, ERM can be indistinguishable from other fish diseases, particularly for those infections associated with *A. hydrophila* and *A. salmonicida* (Austin and Austin, 2007).

As the name suggests, ERM is commonly associated with haemorrhaging of the oral cavity, whereas an absence of this symptom cannot necessarily rule out infection with *Y. ruckeri* (Avci and Birincioglu, 2005, Rucker, 1966, Bush, 1978, Austin and

Austin, 2007). Some ERM symptoms are shown in Fig 1.2. Eyes may protrude from the ocular cavity (= exophthalmia), whereas petechial (= pinprick) haemorrhaging is commonly observed both externally (e.g. around the anus, fins, gills, eyes and lateral body) and internally (e.g. on organs such as the pancreas, liver and swim bladder). Skin may darken due to the development of a granular melanosis. Other physical signs include the inflammation of the intestine (which is often filled with a thick, purulent fluid) and necrosis/liquefaction of internal tissues (Rucker, 1966, Horne and Barnes, 1999, Avci and Birincioglu, 2005, Austin and Austin, 2007). Histological examination of infected material has shown the presence of an acute bacteraemia, particularly in vascularised tissues and in areas of petechial haemorrhaging (Rucker, 1966). With respect to behaviour, fish become anorexic and lethargic, usually swimming near the water surface or retreating to areas with slow currents (Busch, 1983, Rucker, 1966). Some atypical infections associated with *Y. ruckeri* merely result in fish darkening in colour and swimming near the water surface (Frerichs *et al*., 1985).

Table 1.2: Fish species susceptible to *Y. ruckeri* infections (sourced from the Australian Government, Department of Agriculture, Fisheries and Forestry [2008] *Aquatic Animal Diseases Significant to Australia: Identification Field Guide*).

Common name	***Scientific name***
Common species susceptible to infection:	
Atlantic salmon	*Salmo salar*
Brown trout	*Salmo trutta*
Common carp	*Cyprinus carpio*
Goldfish	*Carassius auratus*
Rainbow trout	*Oncorhynchus mykiss*
Other species susceptible to infection:	
Salmonids:	
Arctic char	*Salvelinus alpinus*
Brook trout	*Salvelinus fontinalis*
Chinook salmon	*Oncorhynchus tschawytscha*
Coho salmon	*Oncorhynchus kisutch*
Cutthroat trout	*Salmo clarkii*
Sockeye salmon	*Oncorhynchus nerka*
Non-salmonids:	
Bighead carp	*Aristichthys nobilis*
Burbot	*Lota lota*
Channel catfish	*Ictalurus punctatus*
Cisco	*Coregonus artedii*
Eel	*Anguilla anguilla*
Emerald shiner	*Notemigonus atherinoides*
Minnow	*Pimephales promelas*
Silver carp	*Hypophthalmichthys molitrix*
Sole	*Solea solea*
Sturgeon	*Acipenser baeri*
Turbot	*Scophthalmus maximus*
Whitefish	*Coregonus peled, C. muksun*

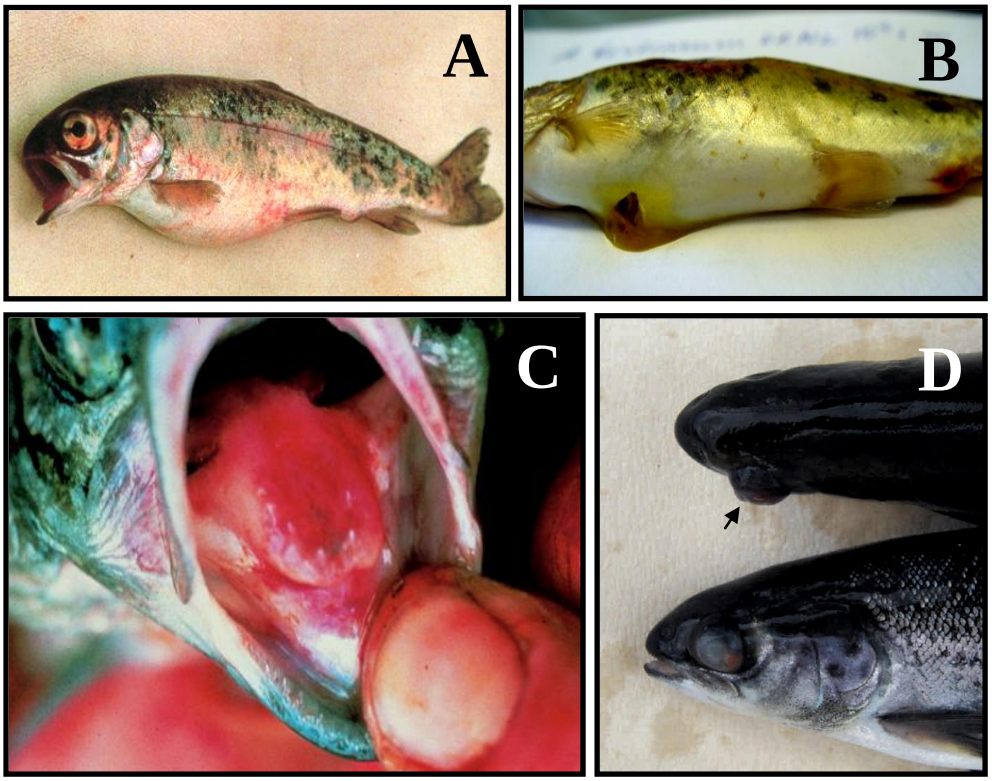


Figure 1.2: Rainbow trout (*Oncorhynchus mykiss*, Walbaum) exhibiting some clinical symptoms commonly associated with enteric redmouth disease. **A:** Fish showing signs of haemorrhaging on both the skin and eyes along with an enlarged stomach (= ascites) due to the accumulation of liquid. **B:** Fish showing signs of generalised necrosis/liquefaction. **C:** Example of haemorrhaging both within and around the oral cavity, thereby giving rise to the term "redmouth". **D:** Trout showing a marked protrusion of the eyeball (= exophthalmia) caused by the accumulation of underlying fluids. Photographs **A**, **C** and **D** sourced from the Australian Government, Department of Agriculture, Fisheries and Forestry (2008) *Aquatic Animal Diseases Significant to Australia: Identification Field Guide*. Photograph **B** courtesy of Professor Brian Austin, Institute of Aquaculture, University of Stirling, Scotland, UK.

1.5 *Yersinia ruckeri*

1.5.1 Phenotypic characteristics

Y. ruckeri belongs to the family Enterobacteriaceae and comprise Gram-negative, asporogenous rods with rounded ends approximately 0.75 µm wide and 1-3 µm long (Ross *et al*., 1966), although morphology can be altered by varying culture conditions (Austin *et al*., 1982). This bacterium grows better at lower temperatures (e.g. 22 to 25ºC) than at higher temperatures (e.g. 35 to 37ºC). Growth may cease at temperatures between 35 to 37ºC when cultivating *Y. ruckeri* on simple media (Ewing *et al*., 1978). Bacterial cells do not possess capsules (Ross *et al*., 1966), whereas some strains are motile by means of peritrichous flagella (Davies and Frerichs, 1989, Ross *et al*., 1966). Biochemically, *Y. ruckeri* isolates share a number of common features (Ross *et al*., 1966, Ewing *et al*., 1978, Frerichs, 1993). For example, all strains produce lysine decarboxylase, ornithine decarboxylase and *β*-galactosidase, but not hydrogen sulphide and indole (Ross *et al*., 1966). Moreover, *Y. ruckeri* is oxidase-negative, nitrate-reductive, and capable of fermenting glucose and mannitol (Ross *et al*., 1966, Ewing *et al*., 1978, Frerichs, 1993). Conversely, sugars such as arabinose, inositol, mannitol, rhamnose or sucrose are not fermented (Ross *et al*., 1966, Frerichs, 1993).

1.5.2 Taxonomy

This organism, originally referred to simply as the "enteric redmouth (ERM) bacterium", is biochemically similar to a *Serratia* or *Yersinia* spp. than any other members of the Enterobacteriaceae (Ewing *et al*., 1978). Taking into account the aggregate chemical reactions given by the ERM bacterium, this organism is biochemically more similar to *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* (Ewing *et al*., 1978). With respect to DNA relatedness, which was measured by hybridizing genomic DNA with $^{32}PO_4$-labelled DNA fragments, this bacterium is 30% similar to both *Serratia* and *Yersinia* spp. Again, this suggests that the organism could belong to either species. On the basis of guanine-plus-cytosine (G+C) content, the ERM bacterium is approx. 47.5 to 48.5%, and thus more akin to that of a *Yersinia* spp. (46 to 48% G+C) as opposed to a *Serratia* spp. (58% G+C). Of interest, it was Ewing *et al*. (1978) who devised the name *Y. ruckeri* in recognition of its isolation by Robert R. Rucker, a researcher who spent many years studying redmouth disease. The final decision to place this organism within the genus *Yersinia* has nonetheless remained controversial, with some researchers

suggesting that the taxonomic position of this organism should be reconsidered (Bercovier *et al*., 1984, Green and Austin, 1983, Kotetishvili *et al*., 2005, Bottone *et al*., 2005).

Although there is a dispute as to what genus the ERM bacterium should belong, the species is genetically homogenous in that strains sourced from a wide geographical area are genetically identical or highly similar (Fernandez *et al*., 2003, Fernandez *et al*., 2007b, Kotetishvili *et al*., 2005, Schill *et al*., 1984, De Grandis *et al*., 1988). Some researchers, such as Schill *et al*. (1984), have suggested that the species is clonal. On the other hand, *Y. ruckeri* strains are serologically heterogenous (see below) and can differ in both morphology (Davies and Frerichs, 1989, Austin *et al*., 1982) and in some biochemical tests (Austin *et al*., 1982, Green and Austin, 1983, Bush, 1978, De Grandis *et al*., 1988, Davies and Frerichs, 1989, Davies, 1991b, Davies, 1991a, Davies, 1990).

1.5.3 Serotyping

Whole-cell serological typing, which includes the recognition of numerous cell surface molecules (e.g. lipopolysaccharides [LPS] and outer membrane proteins [OMPs]) by polyclonal antisera raised in rabbits against specific strains, have shown *Y. ruckeri* to be serologically diverse. The species was originally considered to be comprised of six serovars (I-VI) (Stevenson and Daly, 1982, Ross *et al*., 1966, Bullock *et al*., 1978, Ewing *et al*., 1978, O'Leary, 1977, Stevenson and Airdrie, 1984, Daly *et al*., 1986). The single serovar IV isolate was later removed from the species as it was most likely to be *Hafnia alvei* (De Grandis *et al*., 1988). Later, using a combination of slide agglutination tests and immunoblots against antigenic determinants (e.g. OMPs and LPS), Romalde *et al.* (1993) grouped the species within four serotypes (O1-O4) (Table 1.3). Serotype O1 was sub-divided into two groups; serotype O1a (= serovar I) and serotype O1b (= serovar III). Serovar III isolates were included within the serotype O1 category since they are serologically identical to that of a serovar I (Romalde *et al.*, 1993). Similarly, serotype O2 (= serovar II) was split into three groups (serotype O2a, O2b and O2c), while serotypes O3 and O4 are comprised of serovar V and VI isolates, respectively.

Table 1.3: Relationship between serotypes, subgroups and serovars of *Y. ruckeri* (after Romalde *et al.*, 1993, Tobback *et al.*, 2007).

New serotype	*New subgroup*	*Serovar*	*Designation*
O1	a	I	Hagerman
	b	III	Australian
O2	a, b, c	II	Oregon
O3	NA	V	Colorado
O4	NA	VI	Ontario

NA = Not applicable

The bacterial species can be broadly subdivided into two biotypes depending on their ability to produce a phospholipase and demonstrate motility (Davies and Frerichs, 1989). Thus, biotype 1 (= BT 1) isolates are positive for both motility and phospholipase activity (i.e. Tween 20/80 degradation), whereas biotype 2 (= BT 2) strains are negative for both phenotypes.

Another classification system is one proposed by Davies (1990) based upon the reaction of heat-stable O-antigens with antisera raised in rabbits against inactivated whole-cells. Out of 127 isolates sourced from North America and Europe, five O-serotypes (O1, O2, O5, O6 and O7) could be distinguished. Davies *et al.* (1991b) also differentiated *Y. ruckeri* strains by electrophoretically separating OMPs by molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Isolates were shown to have a 36.5 or 38 kiloDalton (kDa) heat modifiable protein and a peptidoglycan-associated protein with a molecular weight ranging between 36.5 and 40.5 kDa. These workers were subsequently able to identify clonal groups of *Y. ruckeri* using a combination of serotyping, biotyping (BT 1 or 2) and OMP-typing (Davies, 1991a). Hence, serotype O1 strains could be categorised into six clonal groups, two of which (group 2 and 5) being predominately associated with ERM in rainbow trout.

Whereas sorbitol-positive serotype II (= serotype O2b) strains have been found to cause disease in brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*) (Cipriano *et al.*, 1986), they were believed to be confined to North America

and the Baltic countries (Stevenson, 1997). However, isolates of this nature have since caused disease in non-vaccinated rainbow trout reared in Spain (Romalde *et al.*, 2003).

Serotype O1 "Hagerman" strains (= serovar O1a) are highly pathogenic strains for rainbow trout and are the most commonly isolated. These isolates are unique in that they lack the ability to ferment the sugar sorbitol (Ross *et al.*, 1966). Serotype O1 "Hagerman" strains were broadly considered to be motile and capable of producing a lipase, thereby placing these strains within BT 1. Still, the biotyping of "Hagerman" isolates is a topical area for research and one open for further discussion (Section 1.19)

1.5.4 Molecular typing

Whereas serology, morphology and biochemical reactions have proven useful in typing different *Y. ruckeri* strains, they are unable to directly compare the genetic relatedness between isolates. Some molecular techniques used to study *Y. ruckeri* include dot-blot DNA hybridizations (De Grandis *et al.*, 1988), multi-locus enzyme electrophoresis (Schill *et al.*, 1984), multi-locus sequence typing (Kotetishvili *et al.*, 2005) and ribotyping (Garcia *et al.*, 1998). An interesting study by Wheeler *et al.* (2009) using pulsed field gel electrophoresis (PFGE), a technique whereby genomic DNA is digested with a restriction enzyme (in this case *Not*I) and separated on an agarose gel, can be used to differentiate strains. Of the 160 *Y. ruckeri* isolates studied by PFGE, which were sourced from a wide geographical range, 44 profiles or "pulsotypes" could be distinguished. Using PFGE in combination with techniques outlined by Davies (1991a) revealed that serotype O1 isolates, which caused ERM in both the US and Europe, represented a sub-group of similar pulsotypes. In this respect a combination of methods, including both molecular (e.g. PFGE) and serological, can be powerful tools in studying *Y. ruckeri* epidemiology.

1.5.5 Plasmids

A comprehensive study of *Y. ruckeri* plasmids using 183 isolates, sourced from a wide geographical range, was undertaken by Garcia *et al.* (1998). The majority of these strains (89.13%), irrespective of their geographical origin, carried a large plasmid with an apparent size of 75-MDa. Furthermore, this plasmid was confined to strains belonging to serotype O1. As noted by Garcia *et al.* (1998), the presence of a

large plasmid within serotype O1 isolates is not a new feature of this pathogen (Degrandis and Stevenson, 1982, Toranzo *et al.*, 1983, Stave *et al.*, 1987, Guilvout *et al*., 1988, Romalde *et al.*, 1993). In addition to harbouring the 75-MDa plasmid, the majority of strains were also found to possess a plasmid of approx. 12.5 MDa (Garcia *et al.*, 1998), again a feature observed by other workers (Degrandis and Stevenson, 1982, Stave *et al.*, 1987, Romalde *et al*., 1993). On the other hand, some *Y. ruckeri* strains (including those belonging to serotype O1) do not possess plasmids or only carry plasmids with very low molecular weights (Degrandis and Stevenson, 1982, Stave *et al*., 1987, Romalde *et al.*, 1993, Garcia *et al.*, 1998).

Since *Y. ruckeri* belongs to the genus *Yersinia*, and as the majority of virulent serotype O1 strains carry the 75-MDa plasmid, work initially focused on relating this plasmid to those associated with virulence in other *Yersinia* spp. (Degrandis and Stevenson, 1982, Stave *et al*., 1987, Guilvout *et al.*, 1988). For example, using restriction mapping of plasmids with the enzyme *Bam*HI and Southern blotting/hybridization, Guilvout *et al.* (1988) established that the 75-MDa plasmid was not related to the 42-47 MDa plasmid harboured by some *Yersinia* spp. (e.g. *Y. pestis*, *Y. pseudotuberculosis* and *Y. eneterocolitica*).

Still, a potential role for this 75 MDa plasmid in virulence cannot be ruled out since the majority of pathogenic serotype O1 *Y. ruckeri* strains harbour this plasmid. Mendez *et al*. (2009) showed that the 75-MDa plasmid has some sequence identity to the type IV secretion system found within the *Y. ruckeri* chromosome. To what extent this identity extends has still to be determined since work by Mendez *et al.* (2009) primarily focused on the type IV sequence encoded within the *Y. ruckeri* genome.

1.6 *Y. ruckeri* pathogenesis

Bacterial pathogens, both for fish and higher animals (e.g. humans), use specific macromolecules or "virulence factors" to establish infection and disease. This can broadly include molecules associated with bacterial survival outside the host; movement and attachment; invasion of host tissues; evasion of the immune system and aiding systemic spread (Fig 1.3). Whereas each stage of infection and disease can be considered separately, it is important to note that this is a continuous process and that any one feature of virulence could be applied to different stages of disease.

Compared to other fish pathogens (e.g. *A. hydrophila* and *V. anguillarum*), little was known about the molecular basis of *Y. ruckeri* pathogenicity until very recently. It is possible that this lack of research was due, in part, to the successful development and commercialisation of a vaccine (Section 1.17.1). However, recent vaccine failure (Section 1.19) and a sporadic increase in ERM outbreaks has led to a number of excellent studies, as reviewed by Fernandez *et al.* (2007a), regarding *Y. ruckeri* pathogenicity.

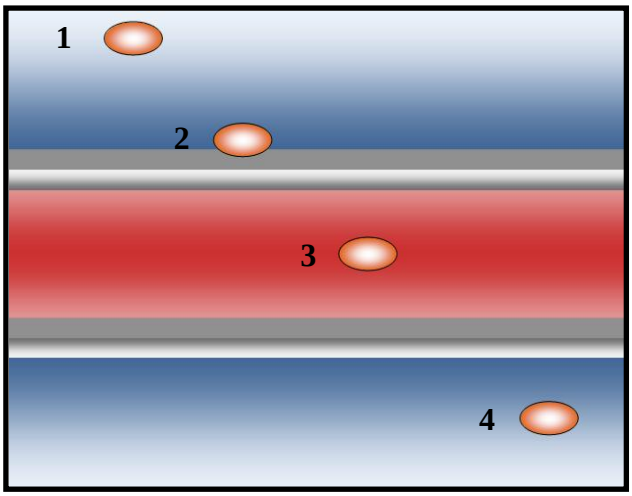


Figure 1.3: Diagrammatic representation of the four main stages of *Y. ruckeri* pathogenicity *in vivo*. This involves bacterial transmission and survival in the environment (**1**); attachment and adherence to host tissues (**2**); infection and systemic spread (**3**); and shedding of bacterial isolates into the environment (**4**). See text for details.

1.7 Transmission and survival

Y. ruckeri is thought to be spread by direct contact (= horizontal transmission) between infected and asymptomatic carriers (Rucker, 1966). Although the spread of this disease to different fish farms is generally considered to be through the import of infected fish (Austin, 2007), cases of ERM have occurred on fish farms which had not recently received new livestock (Bullock *et al.*, 1978). Bush and Lingg (1975) were able to recover the bacterium from the internal organs of asymptomatic fish and suggested that up to 25% of the rainbow trout population could be carrying this

organism within the lower intestine. What is more, the majority of fish surviving ERM infections become carriers of the bacterium. While movement of asymptomatic livestock can contribute to the spread of ERM, it is possible that other animals such as birds, wild fish, invertebrates and even humans may act as vectors (Willumsen, 1989). Hunter *et al.* (1980) found that stressing carrier fish can result in an increase in the periodic shedding of *Y. ruckeri* within faeces, which might persist for some time even after disease has subsided (Rodgers, 1992). This is likely since the occurrence of ERM is generally associated with stressed or overpopulated livestock. The ability to transmit *Y. ruckeri* from the parent to the offspring (= vertical transmission) is questionable since only Sauter *et al.* (1985) has recovered *Y. ruckeri* from non-fertilized Chinook salmon eggs.

Whereas asymptomatic carriers are evidently a major source of infection, *Y. ruckeri* can remain viable for long periods of time within the aquatic environment. For example, bacterial cells can survive for at least 3-4 months in sediment or waters with salinities of 0-20‰ (Thorsen *et al.*, 1992) and have been confirmed to grow *in vitro* under very low saline conditions ($\leq$ 9.0‰) (Altinok and Grizzle, 2001). Additional evidence suggests that stressed and/or nutrient-starved *Y. ruckeri* cells may enter a state of non-culturable dormancy, yet still retain virulence and infectivity (Romalde *et al.*, 1994a). It is possible that the ability of this bacterium to survive for long periods of time within the environment might explain why ERM outbreaks have occurred on sites which had not recently received new livestock (Bullock *et al.*, 1978).

Environmental isolates of *Y. ruckeri* are also capable of forming biofilms, probably with the aid of flagellin or pili, on materials commonly associated with fish farms (e.g. wood) (Coquet *et al.*, 2002a, Coquet *et al.*, 2002b). Susceptibility assays to oxolinic acid demonstrated that sessile cells (those involved in biofilm formation) display a stronger resistance to this antimicrobial compared to their planktonic counterparts. In this respect, biofilms may persist within water tanks even after treatment and thus be a likely source of recurring infections (Coquet *et al.*, 2002b). Proteomic analysis of OMPs by Coquet *et al.* (2005) from immobilised and planktonic cells identified a number of differentially expressed proteins, including some involved in motility and metabolism.

1.8 Adherence and invasion

Attachment and adherence is usually considered to be the first stage in invading the host and establishing infection. Romalde and Toranzo (1993) found that most *Y. ruckeri* strains are moderately adhesive to the salmon cell line CHSE-214. Similarly, Kawula *et al.* (1996) demonstrated that a *Y. ruckeri* strain was highly invasive for fathead minnow (FHM) epithelial cells, yet less so for other cell lines. However, probing the *Y. ruckeri* genome via Southern blotting with gene sequences which are commonly associated with adhesion/invasion in other pathogenic *Yersinia* spp. (e.g. *inv* from *Y. pseudotuberculosis* and *ail* from *Y. enterocolitica*) did not detect any genes with significant sequence identities (Kawula *et al.*, 1996).

Recently, *in vivo* and *in vitro* studies have established that the gills, particularly gill mucus, are major sites of attachment for *Y. ruckeri* cells (Tobback *et al.*, 2009, Tobback *et al.*, 2010a). Moderate levels of bacteria were also found within skin and gut tissues shortly after infection, particularly within intestinal crypts, indicating that bacteria can survive the first line of host defences (Tobback *et al.*, 2009). Moreover, a highly virulent isolate for rainbow trout *in vivo* (Tobback *et al.*, 2009) was later shown to be highly adhesive *in vitro* for gill mucus compared to other isolates (Tobback *et al.*, 2010a), proposing a possible link between virulence and attachment. Further investigation by Tobback *et al.* (2010b) established that attachment/invasion of gill and gut tissue was not significantly different between virulent and avirulent *Y. ruckeri* strains. Consequently, attachment/invasion is not a determining factor of virulence.

Treating bacterial cells with proteases (e.g. pronase and trypsin) and salts which are known to distrupt membrane polysaccharides (e.g. sodium metaperiodate) significantly hindered cell adherence to gill mucus and cultured cell lines, while having little effect on cell viability (Tobback *et al.*, 2010a). Thus, it is likely that both proteins and carbohydrates (e.g. glycoproteins and lectins) have a role in *Y. ruckeri* attachment.

1.9 Systemic spread

In addition to detecting a high number of bacteria attached to the gills shortly after infection, Tobback *et al.* (2009) also observed a large bacterial load within the gills themselves. Since the gills are highly vascularized, it is likely that bacteria

disseminate throughout the underlying capillaries (= septicaemia), eventually leading to colonization of internal organs. Using a green-fluorescent protein (GFP) tagged *Y. ruckeri* strain *in vivo*, Welch and Weins (2005) established that internal organs such as the kidney, spleen and peripheral blood have large bacterial loads following infection.

1.9.1 Resistance to serum-mediated killing

Tobback *et al.* (2010a) discovered that a highly virulent *Y. ruckeri* strain was present in large numbers within internal organs at 72 h post-infection, whereas bacterial numbers for an avirulent strain declined over several days post-infection and did not induce any mortality. It is likely that virulent strains are able to evade the fish immune system, resulting in a severe septicemia/necrosis of internal organs which eventually leads to host death. Tobback *et al.* (2010a) showed that virulent strains *in vivo* (Tobback *et al.*, 2009) were resistant to serum-mediated killing *in vitro* (Tobback *et al.*, 2009), whereas avirulent strains *in vivo* (Tobback *et al.*, 2009) were all serum sensitive *in vitro* (Tobback *et al.*, 2010a). Both studies are in agreement with earlier work by Davies (1991c) which demonstrated that virulent serotype O1 *Y. ruckeri* strains (clonal groups 2 and 5) were resistant to the bactericidal effects of non-immune rainbow trout serum, whereas the majority of avirulent strains were serum-sensitive. Later, Haig *et al.* (2011) confirmed that virulent serotype O1 isolates, which belong to both biotypes and are virulent for rainbow trout, were resistant to non-immune (naïve) rainbow trout serum (PNRTS) and non-immune (naïve) Atlantic salmon serum (PNASS).

Although serotype O1 is usually considered to be the most virulent serotype for rainbow trout, *Y. ruckeri* may exhibit host tropism (i.e. specific strains which cause disease in a specific host species) insofar as some serotype O2 isolates are pathogenic for chinook salmon (*Onchorynchus tshawytscha*) and brook trout (*Salvelinus fontinalis*), but not rainbow trout (Cipriano *et al.*, 1986, Cipriano and Ruppenthal, 1987, Davies, 1991c, Haig *et al.*, 2011). There are nevertheless some exceptions to the notion that all virulent *Y. ruckeri* isolates are resistant to serum mediated killing and vice versa (Haig *et al.*, 2011, Davies, 1991c). For instance, a serotype O2 isolate which was moderately pathogenic for both trout and salmon *in vivo* was sensitive to both PNRTS and PNASS mediated killing. Similarly, a serotype O1 isolate which was resistant to PNASS and PNRTS mediated killing was

highly virulent for salmon, but not for trout (Haig *et al.*, 2011). Hence, while serum resistance is important for virulence, it is nevertheless clear that other important features contribute to the establishment of disease.

It is also worth noting that research on understanding the precise mechanism by which *Y. ruckeri* can resist serum mediated killing is currently very limited. An early study by Furones *et al.* (1990) identified a heat-sensitive factor (HSF) which was produced by virulent serotype O1 strains, but not avirulent isolates. It is possible that the HSF, which was considered as lipid in nature, could contribute to serum resistance. Nonetheless, this is only speculative and requires further investigation. The aforementioned 75-kDa plasmid (Section 1.5.5), which is harboured by most serum-resistant serotype O1 isolates (Guilvout *et al.*, 1988, Davies, 1991c, Garcia *et al.*, 1998), may also be linked with serum resistance. This area should be researched further to gain a greater understanding of serum resistance.

1.9.2 Survival within macrophages

Like other fish pathogens, *Y. ruckeri* has the ability to survive and persist within trout macrophages, both *in vitro* and *in vivo* (Ryckaert *et al.*, 2010a). Visualizing infected macrophages (0 to 24 h post-infection) indicated that bacterial cells were present within phagocytes as autophagocytic vacuoles where they were able to divide and proliferate. Interestingly, no fusion with macrophage lysosomes and bacteria-containing vacuoles was observed, suggesting that internalized bacteria prevent lysosome-mediated killing. Further, internalizing *Y. ruckeri* by trout macrophages did not induce apoptosis, suggesting that this pathogen does not result in cell death.

Virulent *Y. ruckeri* isolates are known to stimulate a respiratory burst by fish macrophages shortly after infection (Stave *et al.*, 1987). Ryckaert *et al.* (2010a) illustrated that reactive oxygen species (ROS) production peaked at 3 h post-infection and was nearly undetectable at 5 h post-infection *in vitro*. Nonetheless, a virulent strain of *Y. ruckeri* was able to withstand ROS-mediated killing *in vitro* since bacterial numbers continued to increase within trout macrophages after 6 h post-infection. Ryckaert *et al.* (2010a) proposed that detoxifying enzymes such as the superoxide dismutase and catalase, which are produced by serotype O1 strains (Horne and Barnes, 1999), could prevent phagocytic killing of internalized bacteria.

Additional *in vivo* analysis by Ryckaert *et al.* (2010a), which is in agreement with data previously obtained by Welch and Wiens (2005), showed that during the early phase of infection the majority of bacterial cells are extracellular. Bacteria then move to become predominately intracellular after the first week of infection. The majority of intracellular bacteria were located within head kidney macrophages (Ryckaert *et al.*, 2010a).

Ryckaert *et al.* (2010a) suggests that the ability of *Y. ruckeri* to invade macrophages could shield against other cell-mediated and humoral immune responses (e.g. lysozyme). Similarly, macrophages are highly phagocytic and long-lived, meaning that they may offer long term protection against the host immune system.

1.10 Extracellular products (ECPs)

Extracellular enzymes or products (ECPs) are secreted by bacterial pathogens to facilitate colonization, aid host cell killing, evade host immune systems and utilize nutrients via the breakdown of macromolecules (e.g. proteins). These enzymes commonly include amylases, proteases, haemolysins, lipases and phospholipases. Work by Romalde and Toranzo (1993) established that *Y. ruckeri* isolates, irrespective of serotyping or virulence, produce moderate levels of ECPs with haemolysin, protease, phospholipase and amylase activities. Administering ECP preparations by injection showed that these enzymes are highly toxic for fingerling rainbow trout; causing signs of necrosis, haemorrhaging and darkening of the skin. On the other hand, LPS was not toxic to fingerling trout since purifying and injecting these molecules did not cause any mortalities.

Since the publication of work undertaken by Romalde and Toranzo (1993), single enzymatic components of the ECPs have been studied in greater detail, particularly those relating to proteolytic and haemolytic activities (Secades and Guijarro, 1999, Fernandez *et al.*, 2002, Fernandez *et al.*, 2003, Fernandez *et al.*, 2004, Fernandez *et al.*, 2007b).

1.10.1 Extracellular protease Yrp1

After protease activity was detected in crude ECP extracts (Romalde and Toranzo, 1993), Secades and Guijarro (1999) varied culturing conditions to determine what effect this may have on protease (= caseinolytic) activity. Their results indicated that

protease production/activity was under catabolic repression. For example, caseinolytic activity increased when grown in peptone or nutrient broth (NB), but was significantly reduced when grown in the presence of casamino acids, carbohydrates (e.g. glucose) or ammonium chloride. Proteolytic activity was also higher at the average temperature of infection (e.g. 17-20°C) when compared to cultures grown at higher temperatures (e.g. 25°C). After purifying native extracellular protein, Secades and Guijarro (1999) found that caseinolytic activity was due to a single 47 kDa alkaline (near pH 8 optimum) Mg^{2+} dependent metalloprotease which is unstable at temperatures above 42°C (i.e. cold-adapted). What is more, some *Y. ruckeri* strains do not produce this protease, thereby allowing strains to be classified as positive (Azo^{+}) or negative (Azo^{-}) for caseinolytic activity.

Further genetic studies by Fernandez *et al.* (2002) found that the *Y. ruckeri* protease gene, termed *yrp1* (*Yersinia ruckeri* protease 1 gene), encodes a protein which shares a high level of homology to a serralysin metalloendopeptidase. Furthermore, DNA sequencing showed that the *yrp1* gene was next to a cluster of genes (*yrpD*, *yrpE* and *yrpF*) involved in the secretion of the Yrp1 protease by means of a type I secretion system. A putative protease inhibitor (*inh*) encoding gene was also shown to be near the *yrp1* gene. To determine the role that the Yrp1 protease has in virulence, site-directed null mutations were introduced into the *yrp1* or *yrpE* genes. Although these mutants were able to grow in nutrient broth, no proteolytic activities were found in the supernatant, nor was the extracellular protease detectable when analysed on a 1D-SDS-PAGE gel. Pathogenicity for both mutant strains was also severely reduced, with LD_{50} values for both mutant strains nearly two orders of magnitude higher than that of the parent strain. These results confirm a role of the Yrp1 protease in establishing disease.

Pertaining that the extracellular protease Yrp1 is secreted by the *Y. ruckeri* bacterium *in vivo*, Fernandez *et al.* (2003) endeavoured to determine what fish matrix or muscle proteins this enzyme may be able to degrade. Proteolytic assays showed that the Yrp1 protease can hydrolyse a wide range of tissue associated proteins, including fibronectin, gelatin and, to a lesser extent, type I collagen, fibrinogen and laminin. With the use of *yrp1::lacZ* fusions, it was shown that expression of this gene was higher at 18°C as opposed to the optimum growth temperature of this bacterium (i.e. 28°C). Expression levels for this protease were

higher in the gills and intestine, and as suggested by the authors, degradation of laminin at these sites may aid systemic spread and invasion of tissues. Furthermore, hydrolysis of basal laminin could cause pores to form within capillary blood vessels, resulting in the leakage of blood through micro-haemorrhages. This might account for the so-called "redmouth" symptom associated with ERM.

Although Azo$^-$ strains of *Y. ruckeri* contain the genes necessary to produce the Yrp1 protease (Fernandez *et al.*, 2003), they do not produce the protease *in vivo* or *in vitro*. Whereas *yrp1* and *yrpE* mutants were severely reduced in virulence towards trout (Fernandez *et al.*, 2002), they could still cause some signs of disease. It is plausible that other ECPs, such as the extracellular haemolysin/cytolysin identified by Romalde *et al.* (1993), may work synergistically with the Yrp1 protease in breaking down host tissues and aiding systemic spread.

1.10.2 Extracellular haemolysin YhlA

Work by Fernandez *et al.* (2004) using *in vivo* expression technology (IVET) identified a putative gene with a high level of identity to a *Serratia*-type haemolysin. Further analysis of this locus identified the presence of two genes, *yhlB* and *yhlA*, which were found to be co-transcribed from a common promoter to make the *yhlBA* operon (Fernandez *et al.*, 2007b). The *yhlB* gene encodes a 561 amino acid protein which is possibly located at the outer membrane and involved in both the secretion and activation of the extracellular haemolysin. The second gene, *yhlA*, encodes a protein with a high sequence identity to a *Serratia*-type pore-forming toxin. Transcriptional analysis of the *yhlBA* operon indicated that expression was higher at lower temperatures (i.e. 18°C) and when grown in iron-limiting conditions, a feature not dissimilar to the expression of the *yrp1* protease gene (Fernandez *et al.*, 2003, Fernandez *et al.*, 2004). Additionally, *yhlA* or *yhlB* mutants were severely reduced in their virulence for fish, with LD_{50} values 10 to 100-fold higher than that of the parent strain. Cytotoxicity for the *yhlA* mutant was likewise reduced compared to the wild-type strain, thus confirming the cytotoxic effects of this enzyme. To determine if the *yhlBA* operon is a conserved feature of the *Y. ruckeri* genome, 12 environmental isolates from a wide geographical area were analysed by PCR to amplify the genes of interest. All strains tested showed the presence of this operon, again confirming the homogenous nature of this bacterial species (Section 1.5.2). As proposed by Fernandez *et al.* (2007b), the pathogen may use the YhlA enzyme to

lyse erythrocytes, thus resulting in the release of intracellular proteins which are bound to iron (e.g. haemoproteins).

1.11 Iron-acquisition and siderophore production

Iron is an essential requirement for most bacterial fish pathogens to proliferate within the host. Although the YhlA haemolysin is used by the pathogen to release intracellular haemoproteins (Fernandez *et al.*, 2007b), other methods associated with iron scavenging and acquisition have been described for this bacterium. Romalde *et al.* (1991) demonstrated that *Y. ruckeri* can grow under iron-limiting conditions and that strains sourced from a wide geographical range representing different serotypes produced phenolate siderophores. Strains were also shown to have a number of OMPs which were inducible under iron-limiting conditions, yet repressed when iron was available in sufficient quantities (Davies, 1991d, Romalde *et al.*, 1991). In addition to identifying genes involved in the excretion of YhlA, Fernandez *et al.* (2004) located genes involved in siderophore-mediated iron uptake. One of these genes, named *rupA*, shared significant identity (52%) to a TonB-dependent receptor from the plant pathogen *Erwinia chrysanthemi*. Other genes identified include those involved in catechol siderophore (ruckerbactin) biosynthesis (*rucC*); ruckerbactin secretion (*rucS*); periplasmic binding of iron-associated ruckerbactin (*rupB*) and its transport to the cytoplasm via an inner membrane permease (*rupDGC* and *exbB*). Gene expression was upregulated when the bacterium was grown in iron-limiting conditions or when grown at lower temperatures. Thus gene expression was not dissimilar to that of other virulence related genes (e.g. *yrp1* and *yhlA*) insofar as temperature and iron availability were influencing factors. Introducing mutations within the *rucC* or *rupG* gene significantly hindered growth *in vitro* and reduced virulence, particularly for *rucC* mutants which had LD_{50} values of at least 100-fold higher than that of the wild-type strain. Evidently, like other fish pathogens, the ability to acquire iron *in vivo* is important for *Y. ruckeri* pathogenesis.

1.12 *In vivo* expression technology (IVET)

In addition to identifying the catecholate siderophore ruckerbactin, and the extracellular YhlA haemolysin/cytolysin (Section 1.10.2), other *in vivo*-induced genes have been identified using IVET by Fernandez *et al.* (2004). For example the *ivi Y. ruckeri* clone *ivi*XII, which exhibited a reduction in virulence towards trout (Fernandez *et al.*, 2004), has since been studied in detail by Mendez *et al.* (2009).

This mutant carried a DNA insertion within a group of putative genes with a shared identity to a type IV exporter. More specifically, these sequences had a significant identity (35 to 58%) and near identical structure to the *traH-I-J-K-C-L-M-N* operon (*tra* operon) located on the pADAP plasmid of *Serratia entomophila*. Interestingly the G+C content of the loci was 53.6%, a value which is slightly higher than that reported for the cumulative genome of *Y. ruckeri* (47 to 48%), suggesting that it was acquired from another bacterial species via horizontal gene transfer. Thus it is possible that the putative *tra* locus within the chromosome of *Y. ruckeri* was acquired from *S. entomophila* (Mendez *et al*., 2009).

Using a *traI* isogenic mutant (*traI*::pIVET8) in an *in vivo* competition assay with the parental strain, Mendez *et al.* (2009) established that the *traI* mutant was attenuated in its ability to grow within the fish. Yet growth *in vitro* under laboratory conditions was not altered compared to the parent strain. Expression analysis of the *Y. ruckeri traI* promoter confirmed that expression was higher at lower temperatures (i.e 18°C), but severely repressed when grown in complex and/or nutrient rich medium. As outlined by Mendez *et al.* (2009), facultative intracellular bacteria such as *Legionella pneumophila* require an intact type IV export system to survive intracellularly. Given that the type IV excretion system in *Y. ruckeri* contributes to growth *in vivo*, it is possible that these genes may have a role in intracellular survival. Further investigation with regard to the role of this type IV secretion system in intracellular survival would be interesting, particularly in light of recent research on this pathogens ability to survive intracellularly (Section 1.9.2).

In addition to possessing a type IV secretion system, it should be noted that *Y. ruckeri* carries a putative type III secretion system (TTSS) within the genome (Gunasena *et al.*, 2003). Nonetheless, its role in virulence has yet to be determined.

Another *in vivo*-induced clone (*Y. ruckeri* 150R*viIX*) described by Fernandez *et al.* (2004) containing a partial ORF with significant homology to a TctC protein of the tripartite tricarboxylate transporter family was later investigated by Navais *et al.* (2011). In this study, an operon involved in the transportation of citrate was identified and named as (*Yersinia* citrate transporter) *yctCBA*. This operon was expressed at higher levels (i.e. 3.5 fold increase) when cells were incubated in media supplemented with citrate, yet repressed in the presence of glucose. Expression was

also higher at the temperature of infection (i.e. 18ºC) as opposed to the optimum growth temperature (i.e. 28ºC). Whereas the *yctCBA* operon does have a physiological role in the uptake of citrate, it does not contribute to virulence as LD_{50} values were not significantly different between the *yctC* mutant or wild-type *Y. ruckeri* 150R strain.

1.13 Signature tagged mutagenesis

Dahiya and Stevenson (2010b) used signature tagged mutagenesis to identify genes which are required for persistence and survival within rainbow trout. Out of 1056 *Y. ruckeri* mini-Tn*5* signature-tagged mutants, 25 were unable to survive within rainbow trout and were significantly reduced in virulence. All mutants of interest were able to grow and survive *in vitro*, suggesting that the mutations possessed by these strains altered their ability to invade tissues and survive. One of these mutants appeared to possess a mutation in the tight adherence locus (Tad) which encodes bundle-forming pili. As discussed by Dahiya and Stevenson (2010b), the Tad locus was previously found by Fernandez *et al*. (2004) to be expressed *in vivo*. Thus, bundle-forming pili may be required for the initial attachment and invasion of host epithelial cells, eventually leading to the systemic spread of the bacterium.

Mutations in other genes associated with the bacterial surface which could contribute to bacterial attachment were also identified by Dahiya and Stevenson (2010b). One of these mutants (termed F1-2) carried a mutation within a gene relating to O-antigen biosynthesis which could, in turn, contribute to the production of LPS. Preliminary analysis of LPS profiles by researchers on both the parent and mutant (F1-2) strain suggests that there are little (if any) in the way of differences between them.

A second *Y. ruckeri* mutant strain (termed C6-1) carried a mutation within a homolog of *znuA*, a gene which encodes a periplasmic protein (ZnuA) involved in the high-affinity zinc transporter ZnuABC. In a similar manner to iron (Section 1.11), zinc is an essential element for survival and can often affect the expression of virulence-related genes. Later, Dahiya and Stevenson (2010c) showed that a *Y. ruckeri* isolate carrying a deletion in the *znuABC* locus (Δ*znuABC*) was severely reduced in virulence compared to the wild-type strain *in vivo*. Additionally, this mutant had nearly 150 to 350-fold lower infection loads in the kidney compared to

the parent strain after 5 days post-challenge. However, the Δ*znuABC Y. ruckeri* strain was not significantly altered in growth compared to the parent strain when cultured in zinc-limiting conditions *in vitro*, suggesting the presence of more than one zinc transporter.

A third STM *Y. ruckeri* mutant (termed F2-4) which was reduced in virulence towards rainbow trout (Dahiya and Stevenson, 2010b) has since been characterised in detail by Dahiya and Stevenson (2010a). This mutant strain had a transposon insertion within a putative UvrY homolog of a two-component system (TCS) known as BarA-UvrY. Dahiya and Stevenson (2010a) determined that the F2-4 mutant was sensitive to H_2O_2 mediated killing and significantly reduced in its ability to invade/survive within epithelial EPC cells. Also, fewer cells of the F2-4 mutant were isolated from the kidney compared to the parent strain after 24 h post-infection. Authors suggested that a reduction in the number of mutant bacterial cells within the kidney could be due to lower invasion capabilities and a heightened sensitivity to oxidative stress (i.e. H_2O_2-mediated killing of phagocytic cells).

1.14 Environmental factors

It is evident that the expression of some virulence factors is higher at lower temperatures and when free iron availability is low. A recent study by Haig *et al.* (2011) recognized that the temperature of infection can alter the virulence of pathogenic strains. For example, a pathogenic serotype O1 *Y. ruckeri* strain was highly virulent (74% mortality) for Atlantic salmon using bath exposure at 16°C, yet significantly reduced in virulence at 12°C (33% mortality). Similar results using a virulent serotype O5 isolate as the challenge strain was also observed by Haig *et al.* (2011). Albeit tempting to assume that the expression of virulence genes (e.g. *yhlA*, *yrp1* and *rucC*) might be elevated at 16°C as opposed to 12°C, other contributing factors such as quorum sensing may have a role in establishing disease.

1.14.1 Quorum sensing

The ability to regulate virulence gene expression with respect to bacterial cell density via quorum sensing (QS) has become an interesting field of study for a number of pathogenic bacteria, including those harmful for fish (Natrah *et al.*, 2011). QS is a form of cell-to-cell communication through the recognition of small signal molecules, commonly acylated homoserine lactones (AHLs), by two-

component systems which ultimately regulate some bacterial phenotypes (i.e. those associated with virulence). In addition to understanding virulence phenotypes with respect to bacterial density/AHL concentrations, identifying so-called quorum sensing inhibitors (QSIs) may prevent disease outbreaks and become an alternative to antimicrobial treatments. This is an attractive method for controlling *Y. ruckeri* infections since this organism has a broad resistance to antimicrobials commonly used in aquaculture (Section 1.16). Preliminary analysis by Bruhn *et al.* (2005) showed that *Y. ruckeri* produces moderate amounts of AHLs, specifically an *N*-3-oxo-octanoyl homoserine lactone (OOHL) and *N*-3-octanoyl homoserine lactone (OHL). What is more, these AHLs are detectable from fish tissues infected with *Y. ruckeri*, suggesting an *in vivo* role for QS.

Y. ruckeri can produce a wide range of AHLs, including eight detectable analogues from over three different AHL classes (Kastbjerg *et al.*, 2007). The most predominant AHL produced by *Y. ruckeri* is 3-oxo-C8-HSL (Kastbjerg *et al.*, 2007). In an attempt to determine what role QS may have in regulating virulence, Kastbjerg *et al.* (2007) endeavoured to relate Yrp1 protease production with different AHLs and QSIs. However, after testing different AHLs or QSIs at various concentrations under different culturing conditions, production of the extracellular protease was generally not altered by these chemicals. In this respect, preliminary data implies that this virulence factor was not under QS regulation. This is currently the only published study to date which attempts to correlate QS to virulence phenotypes of *Y. ruckeri*.

Genes potentially involved in *Y. ruckeri* quorum sensing were identified by Temprano *et al.* (2001) by constructing a genomic library from a virulent *Y. ruckeri* strain in *E. coli* and streaking against *Chromobacterium violaceum* to test for the production of violacein. This is a dark, purple pigment which is produced by *C. violaceum* and is indicative of AHL production. One of the *E. coli* transformants, which stimulated violacein production, was shown to possess a genomic *Y. ruckeri* fragment containing two open reading frames (ORFs) with shared homology to genes encoding the LuxR/LuxI protein family. The first *Y. ruckeri* ORF, termed *yruR*, encoded a predicted protein of 247 amino acids (aa) and shared significant homologies to that of a transcriptional activator originally identified in *V. fischeri*. The second ORF (termed *yruI*) encoded a predicted protein of 217 aa which is

potentially involved in AHL synthesis. Even though these genes proved useful in detecting *Y. ruckeri* in a PCR reaction, their role in virulence was not deduced. It would be interesting to construct mutations within the *yruR*/*yruI* genes to determine if this has any effect on pathogencicty *in vivo*. Nonetheless, the identification of AHL production by *Y. ruckeri in vivo* implies that QS has a role in virulence (Bruhn *et al.*, 2005).

1.15 Diagnosis and detection of *Y. ruckeri*

Given the number of symptoms associated with ERM (Section 1.4.2), and since some fish may be asymptomatic carriers of disease, diagnosis cannot be based on clinical symptoms alone. Other methods of identification and detection must therefore be used.

1.15.1 Bacteriological and serological detection

Fish are usually killed to recover *Y. ruckeri*. Non-lethal methods such as faecal analysis (Hunter *et al.*, 1980, Rodgers, 1992, Busch and Lingg, 1975) and kidney-biopsy (Noga *et al.*, 1988) are available for diagnosing rare and/or valuable fish. Generally, the organism can be recovered from infected tissues and organs (e.g. kidneys) and cultured on a variety of growth media including tryptone soya agar (TSA) (Stevenson and Daly, 1982); nutrient agar (NA) (Secades and Guijarro, 1999); brain heart infusion agar (Arias *et al.*, 2007); Columbia blood agar (Gibello *et al.*, 2004) and MacConkey agar (Gibello *et al.*, 1999). Selective media are available for culturing *Y. ruckeri* (Furones *et al.*, 1993, Waltman and Shotts, 1984), although they are not routinely used in diagnostics (Dr D. Austin, Heriot-Watt University, *personal communication*).

When culturing *Y. ruckeri* on TSA and NA at 20-28°C for 48 h; raised, circular and opaque colonies of 2 to 3 mm in diameter develop (Ross *et al.*, 1966, Bullock *et al.*, 1978). Biochemical characteristics discussed earlier in Section 1.5.1 can be used to identify the pathogen. The commercialisation of biochemical test kits, such as the API 20E rapid identification system, has significantly improved the ease of diagnosis. Still, API 20E test strips may be unable to differentiate *Y. ruckeri* from some other enterics, namely *Hafnia alvei* (Austin *et al.*, 2003). Serological methods, such as an enzyme linked immunosorbent assay (ELISA) (Austin *et al.*, 1986) and an immunofluorescence antibody technique (IFAT) (Smith *et al.*, 1987) have been

developed to recognise some *Y. ruckeri* isolates. As outlined by Bullock and Cipriano (Bullock and Cipriano, 1990), polyclonal antibodies cannot react with all known serotypes. Thus negative or weak reactions with antibodies cannot necessarily rule out the possibility of *Y. ruckeri*. For instance, Austin *et al.* (2003) showed that polyclonal antisera raised in rabbits against inactivated *Y. ruckeri* cells agglutinated only weakly with isolates sourced from fish showing signs of ERM. It was only when molecular methods, such as the sequencing of 16S ribosomal RNA (rRNA) was performed, that strains were in fact identified as *Y. ruckeri*.

1.15.2 Molecular methods of diagnosis and detection

Although bacteriological and serological methods can be used to identify *Y. ruckeri*, these methods are often laborious, costly and time consuming. Alternatively, polymerase chain reaction (PCR) can be used to rapidly and specifically identify *Y. ruckeri*, both in artificially and naturally infected tissues, using primers which target non-conserved rRNA sequences (Altinok *et al.*, 2001, Gibello *et al.*, 1999). Other molecular methods of detection include multiplex PCR (Del Cerro *et al.*, 2002, Altinok *et al.*, 2008, Onuk *et al.*, 2010), reverse-transcriptase PCR (Wilson and Carson, 2003, Glenn *et al.*, 2011, Keeling *et al.*, 2012), infrared spectroscopy (Wortberg *et al.*, 2012) and a highly sensitive loop-mediated isothermal amplification (LAMP) assay (Saleh *et al.*, 2008). The latter test, which was optimised to amplify the *Y. ruckeri* QS genes *yruR*/*yruI* described by Temprano *et al.* (2001) (Section 1.14.1), is of particular interest in that it is fast and does not require extensively trained personnel or specialist equipment. In principle, only a water bath and a set of primers are required to perform a LAMP reaction. Whereas molecular based methods have advanced considerably within the past 20 years, it is worth noting that they are not usually used as standalone methods of diagnosis. Generally, a combination of both molecular and more traditional diagnostic methods (e.g. biochemical tests) are used for identification (Austin *et al.*, 2003).

1.16 Treatment and control methods

Once ERM occurs in livestock, the options for treatment are somewhat limited. As outlined by Austin and Austin (2007), antimicrobials such as quinolones and sulphamerazine (Rucker, 1966), tribrissen and tiamulin (Bosse and Post, 1983), potentiated sulphonamides (Bullock *et al.*, 1983), and oxolinic acid (Rodgers and Austin, 1983) have been used with some success to alleviate ERM symptoms. The

use of antimicrobials is often a contentious issue since it has led to the development and spread of antibiotic resistance genes (Degrandis and Stevenson, 1985, Gibello *et al.*, 2004). Antimicrobials aside, there are very few other alternatives but to impose movement restrictions and cull infected livestock when disease occurs. Prophylaxis in aquaculture is often considered to be more important than treatment, with a number of preventative measures now available to avert disease (Austin and Austin, 2007).

1.16.1 General prophylaxis

As with many opportunistic fish pathogens, the occurrence of ERM is usually linked with immunocompromised livestock due to inadequate husbandry (i.e. high stocking densities and unsanitary waters) (Austin and Austin, 2007, Tobback *et al.*, 2007). Reducing stocking densities, sterilizing equipment (e.g. ponds and utensils) and practising good feeding regimes can considerably reduce the likelihood of disease outbreaks. Selective breeding of fish, with a focus on increasing the efficiency of the host's innate immune system, has also been considered to decrease host susceptibility to *Y. ruckeri* infection (Austin and Austin, 2007). A notable study by Hadidi *et al.* (2008) demonstrated that a larger spleen significantly increased resistance to challenge with *Flavobacterium psychrophilum*, but not to *Y. ruckeri*, suggesting that resistance is pathogen-specific. Although unsuccessful in terms of protecting against ERM, it is nevertheless a step forward in determining factors associated with selective breeding and increased resistance to *Y. ruckeri* infection.

1.16.2 Probiotics and immunostimulants

In addition to adequate husbandry and feeding regimes, probiotics have been successfully used in aquaculture or in an a laboratory environment to prevent ERM (Abbass *et al.*, 2010, Kim and Austin, 2006, Brunt *et al.*, 2007, Raida *et al.*, 2003, Capkin and Altinok, 2009, Sica *et al.*, 2012). Probiotics are broadly defined as "living microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO; World Health Organization). For example, supplementing rainbow trout feed with *Carnobacterium* spp. (e.g. *C. divergens* and *C. maltaromaticum*) increased resistance to challenge with a highly virulent *Y. ruckeri* strain (Kim and Austin, 2006). Fish treated with these probiotics had significantly heightened phagocytic activity for head kidney macrophages and increased lysozyme activities for serum and gut mucus. Hence, it is possible that

probiotics stimulate the innate immune system, thereby priming the host against bacterial infection. Probiotics may also be beneficial for the host insofar as they could produce inhibitory compounds and/or compete with the pathogen for nutrients and adhesion sites (Tobback *et al.*, 2007).

In addition to probiotics, administering other compounds in the diet can have a positive effect on health and/or elicit immunostimulatory effects which may be beneficial in combatting *Y. ruckeri* infections. These include algal extracts (Gioacchini *et al.*, 2008, Sheikhzadeh *et al.*, 2012), vitamins (Furones *et al.*, 1992), oestrogens (Wenger *et al.*, 2011), and β-mercaptoethanol treated yeast cells (Tukmechi *et al.*, 2011). An interesting study by Ryckaert (2010b) showed that a non-lethal heat shock (NLHS) for platyfish (*Xiphophorus maculatus*) increased the production of heat shock proteins (HSPs) which could stimulate the immune system and increase survival against challenge with *Y. ruckeri*. When recombinant HSPs from *E. coli* were administered to fish, in addition to NLHS, levels of survival after challenge slightly increased (20%). For bacterial HSPs to be an economically viable vaccine, further research must be carried out before it can be applied on an industrial scale.

Overall, though good husbandry in combination with other non-specific techniques (e.g. probiotics) can significantly reduce the possibility of an ERM outbreak, sporadic cases still occur due to the endemic nature of *Y. ruckeri*. This necessitates the development of more specific methods (i.e. vaccines) in an attempt to prevent outbreaks of disease.

1.17 Vaccines

Vaccine development has become crucial in recent years for preventing many fish diseases, including ERM (Stevenson, 1997). The nature of vaccines are varied and can broadly include inactivated whole cells, live attenuated bacteria, purified protein sub-units, and recombinant (DNA/protein) vaccines. Nonetheless, the application of a formalin-inactivated whole cell (= bacterin) vaccine has generally prevented ERM with some success.

1.17.1 Bacterin vaccines

Work to produce a successful bacterin vaccine can be linked back to the work of Ross and Klontz (1965). These workers found that orally administering phenol-inactivated bacterial cells mixed with feed resulted in high levels of protection (90% survival) against challenge with a virulent *Y. ruckeri* strain. Later studies demonstrated that inactivated preparations can be administered to fish either orally, by injection, immersion (fish are bathed in a diluted preparation), showering, spraying, and via anal intubation (Ross and Klontz, 1965, Anderson and Nelson, 1974, Cossarini-Dunier, 1986, Johnson and Amend, 1983a, Johnson and Amend, 1983b). Although injection is superior for conferring protection against artificial challenge with *Y. ruckeri* (Johnson and Amend, 1983a, Vignuelle, 1990, Anderson and Nelson, 1974), this method is impractical for large-scale vaccinations and is generally reserved for large and/or valuable fish (Austin and Austin, 2007). Nevertheless, McCarthy and Anderson (1982) showed that vaccinating fish against a sorbitol-negative serotype I *Y. ruckeri* isolate widely cross-protected against challenge with other serotype I isolates. Vaccinating fish with a sorbitol-negative serotype II strain (Cipriano *et al.*, 1986), or indeed a serotype I isolate, protected fish against challenge irrespective of serotypes (Cipriano and Ruppenthal, 1987).

It is worth noting that although the bacterin vaccine has generally offered a high level of protection against ERM, it has recently failed to protect against an emerging strain of *Y. ruckeri* (Section 1.19). A new formulation of inactivated bacteria is now commercially available which offers some level of protection against this new strain. Still, outbreaks of this kind will inevitably occur again in vaccinated fish, thereby necessitating the development and/or improvement of next generation vaccines.

1.17.2 Attenuated vaccines

The use of attenuated bacteria as a vaccine is believed to have a number of benefits over traditional bacterins as they elicit a stronger cell-mediated response (Marsden *et al.*, 1996) and can mimic natural infection (e.g. express stress-related proteins), yet not lead to the diseased state. Temprano *et al.* (2005) developed a live, attenuated *Y. ruckeri* vaccine by insertionally inactivating the *aroA* gene via allelic replacement with a DNA fragment containing a kanamycin resistance (Km^R) marker (*aroA*::Km^R). The *aroA* gene, which encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, is essential for producing chorismic acid *in vivo*, a prerequisite

for synthesizing p-aminobenzoic acid, aromatic amino acids and folate. Actually, *aroA*::Km^R *Y. ruckeri* mutants were significantly attenuated in virulence for fish and were not recoverable from organs 48 h post-vaccination. Intraperitoneal (i.p.) vaccination with this mutant also conferred excellent protection against challenge with the wild-type *Y. ruckeri* strain (RPS = 87.5-93.75%). Although the DNA insertion within the *aroA* gene was deemed stable, use of an attenuated vaccine is often met with some criticism for fear of reverting back to virulence (Austin and Austin, 2007). Moreover, recombinant bacteria of this nature can be shed from vaccinated fish and thus result in environmental contamination (Vivas *et al.*, 2004). It should also be noted that stigma surrounding live vaccines, even if they are deemed safe for use, is often still met with criticism in the aquaculture industry (Professor B. Austin, Stirling University, *personal communication*).

1.17.3 Protein sub-unit vaccines

Fernandez *et al.* (2003) investigated the immunogenicity of the extracellular protease Yrp1 (Section 1.10.1) by heat-inactivating the enzyme (= toxoid) and administering to fish as a vaccine. Initial results suggested that i.p. injection of this toxoid gave excellent protection (95% survival) against *Y. ruckeri* infection. Although not directly stated by the author, the challenge strain (*Y. ruckeri* 150) was previously shown to be an Yrp1 producing (Azo^+) isolate (Secades and Guijarro, 1999). Should protection be correlated to the production of an anti-Yrp1 antibody, it is therefore plausible that this vaccine may not confer protection to challenge with an Azo^- strain. Even so, this is the first (and currently only) toxoid to successfully protect against *Y. ruckeri* infection when used as a vaccine and thus merits further attention. For instance, cloning/overexpression of the *yrp1* gene might allow the toxoid to be produced on a large scale, although this is only speculative and undoubtedly requires further investigation.

1.17.4 Recombinant vaccines

This area of vaccine development has been negligible for ERM. Most DNA vaccines have been researched for conferring protection against fish viruses. For example, trout vaccinated with plasmid DNA encoding the viral haemorrhagic septicaemia virus (VHSV) or the infectious haematopoietic necrosis virus (IHNV) glycoprotein genes, were relatively protected against VHSV or IHNV challenge. In contrast, fish were not significantly protected against challenge with *Y. ruckeri* (Lorenzen *et al.*,

2002). It is likely that specific anti-viral mechanisms are involved in conferring protection against viruses which cannot protect against bacterial pathogens.

As of 2012, there is currently no recombinant protein or DNA based vaccine described within the literature which successfully protects against ERM.

1.18 Immune response to *Y. ruckeri* infection

It is likely that the development of new vaccines will depend on a greater knowledge of the fish immune system and how the host responds to bacterial infection. Like terrestrial vertebrates (e.g. humans), fish possess both a non-specific (= innate) and adaptive immune system, which may be broadly categorised as being cellular or humoral in nature. However, despite an extensive amount of research, features of the fish immune system which aid in preventing the development of ERM are still speculative; it is likely to involve both an innate (below) and adaptive immune response (Section 1.18.2).

1.18.1 Innate immune response

The innate immune system is usually considered to be the primary defence of fish against pathogens (Ellis, 2001). Indeed, this aspect of the fish immune system is thought to be extremely important in preventing fish pathogens. Unlike an adaptive immune response, the innate immune system is non-specific (i.e. not dependent upon the distinct recognition of molecules associated with any one pathogen) and is usually very fast in responding to infection. Research to date has shown that *Y. ruckeri* infection can result in the upregulation of genes which encode proteins related to an innate immune response. These generally include cytokines (e.g. TNF-a, IL-1β, IL-8, IL-10 and IL-22); Toll-like receptors (e.g. TLR5); acute phase proteins (SAA, hepcidin, transferrin and precerebellin); antimicrobial peptides (e.g. cathelicidin-2); nitric oxide synthase; mannose binding lectins and complement factors (e.g. C3, C5 and factor B) (Chettri *et al*., 2012, Ruangsri *et al*., 2012, Bridle *et al*., 2011, Monte *et al*., 2011, Raida *et al*., 2011a, Diaz-Rosales *et al*., 2009, Raida and Buchmann, 2009, Harun *et al*., 2011, Evenhuis and Cleveland, 2012). Vaccinating fish against formalin-inactivated bacterial cells similarly resulted in the upregulation of genes related to innate immunity (Raida and Buchmann, 2008a, Raida and Buchmann, 2007). However, the majority of these studies concerning the response of the fish innate immune system to *Y. ruckeri* were performed over a

relatively short period of time (0 to 3 months); thus the long term immunological responses to bacterial infection following vaccination, or a previous encounter with this bacterium, cannot be determined from these data. This would probably concern the adaptive immune system, of which its role in protecting against ERM has only recently been approached within the published literature.

1.18.2 Adaptive immune response

An adaptive immune response towards *Y. ruckeri* was first noted by Raida and Buchmann (2008b). This study showed that genes involved in innate immunity (e.g. IL-1β) were up regulated in naïve fish following *Y. ruckeri* infection. After 35 days post-challenge, trout surviving the primary infection were re-infected with the bacterial pathogen. Unlike the primary infection whereby mortality reached 37%, only 7% of fish that were re-infected with the pathogen died. Bacterial clearing was also increased within the spleen of re-infected fish, although the expression of genes related to innate immunity had decreased to levels not dissimilar to that of control fish (i.e. fish not challenged or vaccinated). This was considered by the authors to be indicative of an adaptive immune response (Raida and Buchmann, 2008b).

More recently, Deshmukh (2012) advocated that vaccine-induced protection against *Y. ruckeri* over a longer period of time (4 to 8 months) involves an adaptive immune response. What is more, Raida *et al*. (2011b) showed that plasma antibodies against *Y. ruckeri* increased after 8 and 12 weeks post-vaccination. This level of antibody production was also correlated with levels of protection, hence indicating that an adaptive immune response is involved in conferring protection. Taking results by Raida *et al*. (2011b) into consideration, it is likely that the role of the adaptive immune system will become a topic of further research in the near future.

1.19 EX5: a new problem for aquaculture

In 1991, it was reported that a site in southwestern England had suffered a severe loss of vaccinated rainbow trout. When researchers arrived at the farm, fish were found to be severely overstocked in unsanitary waters (Professor B. Austin, University of Stirling; *personal communication*). In fact the situation was so uncontrollable that the fish farm in question was later closed. The aetiological agent of disease was later attributed to a bacterium with similarities to both *Hafnia alvei* and *Y. ruckeri*. Correctly identifying the bacterium initially posed a problem insofar as strains were non-motile, phospholipase negative and positive for the Voges-Proskauer (VP) reaction; phenotypes which were not usually attributed to *Y. ruckeri* (Austin *et al.,* 2003). Nevertheless, sequence analysis of 16S rRNA encoding genes confirmed that isolates were in fact *Y. ruckeri* (100% homology). Strains of a similar nature have since caused disease in vaccinated livestock throughout Europe and the USA (Strom-Bestor *et al.*, 2010, Wheeler *et al.*, 2009, Arias *et al.*, 2007, Fouz *et al.*, 2006).

The non-motile nature of some *Y. ruckeri* strains was not previously unknown (Davies and Frerichs, 1989), although the majority of disease outbreaks up until the 1990s were attributed to traditional "Hagerman" (= biotype 1 [motile], serotype O1) strains. Whereas Austin *et al.* (2003) considered these strains to be part of a new biogroup, others considered them to be biotype 2 (= non-motile) serotype O1 isolates (Arias *et al.*, 2007, Davies and Frerichs, 1989, Strom-Bestor *et al.*, 2010, Fouz *et al.*, 2006, Wheeler *et al.*, 2009, Evenhuis *et al.*, 2009, Welch *et al.*, 2011, Welch, 2011). All workers thus far agree that the non-motile isolates belong to the serotype O1 as they are unable to ferment sorbitol (Arias *et al.*, 2007, Davies and Frerichs, 1989, Austin *et al.*, 2003, Fouz *et al.*, 2006, Evenhuis *et al.*, 2009) and agglutinate slightly better with polyclonal antibodies raised against serotype O1 cells compared to serotype II (Austin *et al.*, 2003). LPS from these strains also react with antiserum raised against serotype O1 when used in an immunoblot (Fouz *et al.*, 2006). In addition to being non-motile, reoccurring isolates causing disease in the vaccinated host share a number of biochemical features which differ from the original "Hagerman" strains.

Terminology can often be misconstrued when referring to different *Y. ruckeri* strains, particularly when standardisation has yet to be accepted by researchers.

Hence for the purposes of this study, non-motile strains with phenotypes outlined in Table 1.4 will be collectively known as "EX5". The original motile isolates will be collectively referred to as "serotype O1". Although the author accepts that so-called "EX5" strains belong to serotype O1, they do not match the original definition of this serotype (i.e. motile, phospholipase positive and VP negative). Therefore, when the term serotype O1 is used in this study it refers to the original "Hagerman" strain in Table 1.4.

Table 1.4: Biochemical and phenotypic comparisons between two biotypes of *Y. ruckeri* (after Austin *et al.*, 2003).

	Biotype 1 (O1)	***Biotype 2 (EX5)***
Motility	+	-
Tween 20/80 degradation	+	-
VP reaction	-	+

Despite the research on EX5 *Y. ruckeri* isolates a number of unanswered questions remain. One essential question surrounding EX5 is why it is able to cause disease in the vaccinated host, since the strain used in the vaccine preparation is essentially the same bacterial species. Similarly, serological testing by Austin *et al.* (2003) showed that antibodies raised in rabbits against inactivated serotype O1 cells weakly agglutinated EX5, yet reacted strongly with a serotype O1 strain. Again, it is perplexing why the EX5 isolate did not agglutinate with polyclonal antibodies since both strains used in the agglutination assay are essentially the same bacterial species and serotype. Thus the majority of antigens presented by the serotype O1 cell should be very similar, if not identical, to that of the EX5. As noted by Tobback *et al.* (2007), the lack of information regarding the immunological response of fish to vaccination and what *Y. ruckeri* antigens/molecules are involved in conferring protection complicates the matter further.

Unlike serotype O1 isolates, EX5 strains are non-motile and apparently unable to produce flagellin (Evenhuis *et al.*, 2009). It has been suggested on numerous occasions that vaccinating fish against serotype O1 strains might have exerted a

selection pressure on flagellin. In this respect, hosts vaccinated with a flagellin-producing strain would be susceptible to infection by a strain which is not producing this molecule. Again, even if flagellin is not produced by EX5 strains, it is conceivable that other differences exist between them. Therefore, it would be interesting to determine if any proteins are differentially expressed between the serotype O1 and EX5 isolates.

1.20 Aims of the project

1. Evaluate the protective role of *Y. ruckeri* flagellin as a component of a whole-cell-vaccine and as a purified (= sub-unit) vaccine.

2. Identify potential virulence factors which are contributing to an increase in EX5 pathogenicity using proteomic and transposon mutagenesis techniques.

3. Investigate the role, if any, which overexpressed proteins produced by EX5 isolates have towards virulence.

CHAPTER 2: MATERIALS AND METHODS

2.1 General methods

2.1.1 Materials

Unless otherwise stated in the text, chemicals and reagents were purchased from Sigma-Aldrich (UK) or Fisher Scientific (UK). All chemicals, enzymes and other materials were stored and handled in accordance with the manufacturers' instructions.

2.1.2 Centrifugation

Small samples were centrifuged in a 1.5 ml Eppendorf tube using a Micro Centaur microfuge (MSE). The MSE centrifuges were run at room temperature or 4°C as appropriate and reached a maximum speed of 13,400 x *g*. Larger volumes, however, were centrifuged in a Beckman Avanti J26 XP centrifuge.

2.1.3 Bacterial strains, antibiotics and storage conditions

Compositions of all media used to cultivate bacteria are shown in the appendix (Table 5.1). All bacterial strains and vectors used in this study are outlined in Table 2.1. A diagram of the pET28-b plasmid used in this research is available in the appendix (Fig 5.1), as is a map of the pRL27 vector (Fig 5.2). *Y. ruckeri* isolates were originally sourced from dead or moribund rainbow trout (*Oncorhynchus mykiss*, Walbaum) by Professor B. Austin and for this work were obtained from the fish pathogen collection of the School of Life Sciences, Heriot-Watt University. *Y. ruckeri* cultures were routinely grown on tryptone soya agar (TSA) plates or in tryptone soya broth (TSB) with incubation at 28°C or 16°C for 18-72 h. *E. coli* isolates, however, were grown on nutrient agar (NA) or Luria-Bertani agar (LA) plates at 37°C for 18-72 h. For liquid cultures, single colonies of *E. coli* were grown in nutrient broth (NB) or Luria-Bertani (LB) broth at 28°C or 37°C for 18 to 72 h. Heat labile chemicals (i.e. antibiotics) were sterilised by filtration using 0.22 µm porosity filters (Millipore). When required, antibiotics were added to media after autoclaving (121°C, 15 min) and cooling (~50°C) in the following concentrations; kanamycin (50 µg/ml), nitrofurantoin (5 µg/ml) and streptomycin (20 µg/ml). Iron-deficient medium was prepared by the addition of 2'2'-bipyridyl (100 µM/ml) after washing glassware once with 1 M HCl and then several times in dH_2O prior to

autoclaving. Stock cultures of bacteria were stored at −70°C in TSB, NB or LB broth supplemented with 30% (v/v) sterile glycerol.

Table 2.1: Bacterial strains and plasmids used in this study

Name	*Characteristics/Uses*	*Reference/Source*
Y. ruckeri		
BA19	Serotype O1, biotype 1 (motile)	This study
YR1	-	Austin *et al.* (2003)
EX5	Serotype O1, biotype 2 (non-motile)	-
R1	-	-
O2BA2	Serotype O2, biotype 1 (motile)	This study
O3BA3	Serotype O3, biotype 1 (motile)	-
O4BA4	Serotype O4, biotype 1 (motile)	-
E. coli		
BL21-DE3	Overexpressing recombinant proteins	Stratagene, UK
XL1-Blue	Maintaining recombinant plasmids	-
DH5α/λ*pir*	Cloning transposon inserts as *pir*-dependent plasmids	Miller and Mekalanos (1988)
BW20767	Donor strain for conjugation experiments	Metcalf *et al*. (1996)
Plasmids		
pRL27	Suicide vector possessing transposon, Km^R	Larsen *et al*. (2002)
pET28-b	Gene cloning for overexpression	Invitrogen, UK

2.1.4 Morphological, biochemical and phenotypic characterization of *Y. ruckeri* isolates

Strains considered to be *Y. ruckeri* were verified following procedures outlined by Austin and Austin (2007).

2.1.4.1 Micro-morphology

Overnight cultures on TSA plates were used to study micro-morphology. Thus, a cell suspension in 0.9% (w/v) saline was spread over a sterile glass microscope slide and stained by Hucker's modification of the Gram-stain (Hucker and Conn, 1923) before examining under a Kyowa light microscope (magnification = x1000). In

addition to the Gram-stain, colonies on plates were examined visually to determine cell morphology, arrangement, shape and colour.

2.1.4.2 API-20E rapid identification system

Isolates were examined using the substrate utilization API 20E (Bio-Mérieux, UK) rapid identification system following the manufacturer's instructions, albeit with an incubation period for 48 h (as opposed to 24 h) and at a temp of 25°C instead of 37°C (Austin *et al.*, 2003). Species were identified by entering the API-20E data into the manufacturers' computer-based identification system.

2.1.4.3 Oxidase and catalase production

A piece of Whatman filter paper (No. 1) was moistened with 1% (w/v) N,N,N',N'-tetramethyl-*p*-phenylenediamine dihydrochloride before streaking the surface with a single colony grown overnight on TSA at 28°C. A positive reaction for oxidase was recorded visually by the development of a purple pigment within 30 seconds. The ability to produce catalase was examined by adding a drop of 5% (v/v) H_2O_2 solution to a bacterial colony on a glass slide. If rapid evolution of bubbles entailed within a few seconds, due to oxygen being released from the breakdown of H_2O_2, then strains were considered positive.

2.1.4.4 Motility assays

Motility was determined by stab inoculating a single bacterial colony into the bottom of motility plates (Columbia broth supplemented with 0.3% [w/v] Bacteriological agar No 3, average depth = 3 mm) before incubating at 28°C for 24 h (Coquet *et al.*, 2002, Evenhuis *et al.*, 2009). Strains were considered to be motile if migration (= swimming) zones were visible from the point of inoculation after incubation. In addition to using semi-solid agar, swimming ability was observed using light microscopy (magnification of x400) while taking water movement and Brownian motion into consideration.

2.2 Protein methods

2.2.1 Determination of protein concentrations

Protein concentrations were measured using the protein assay based on the Bradford dye binding procedure (Bradford, 1976) using a standard curve based on bovine serum albumin (BSA).

2.2.2 Protein purification buffers and storage conditions

Autoclaved Millipore® dH_2O was used to make buffers for protein analysis. All buffers used for protein purifications were sterilized by filtration and stored throughout on ice to prevent protein proteolysis.

2.2.3 Preparation of whole cell proteins (WCPs) for 1D and 2D SDS-PAGE

WCPs were purified following a modified French press lysis method recommended by Cull and McHenry (1990). After growing bacterial cultures (Section 2.1.3), cells were harvested by centrifugation (4°C, 9,605 x *g*, 30 min) and resuspended in 10 ml of cell lysis buffer A (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid [EDTA], 1% (w/v) dithiothreitol [DTT], 2 mM phenylmethanesulphonylfluoride [PMSF]). After storing on ice for 20 min, the suspension was passed through a pre-chilled French pressure cell press (3 repeats at 14,000 psi) to lyse cells. Samples were again stored on ice (20 min) before removing cellular debris and unbroken cells by centrifugation (4°C, 20,442 x *g*, 30 min). The supernatant was then precipitated using 10% (w/v) TCA in acetone (Section 2.2.9).

2.2.4 Isolation of outer membrane proteins (OMPs) for 1D SDS-PAGE

Outer membrane fractions were prepared using a method previously described by Chart and Trust (1983). After culturing bacteria, cells were harvested by centrifugation (4°C, 9,605 x *g*, 30 min) and gently washed in 20 ml of 200 mM Tris-HCl (pH 7.5). Cells were again harvested (4°C, 7,741 x *g*, 20 min) before re-suspending in 10 ml of ice-cold OMP lysis buffer (200 mM Tris-HCl [pH 7.5], 50 mM NaCl, 10 mM EDTA, 2 mM PMSF). The suspension was disrupted on ice by sonication using 4 x 60 s bursts with 30 s cooling periods. Unbroken cells were removed by centrifugation (4°C, 20,442 x *g*, 30 min), whereas the cell envelope fraction was pelleted by high-speed centrifugation (10°C, 75,600 x *g*, 2 h). To selectively solubilise the cell membrane, the pellet was re-suspended in 5 ml of 0.5% (w/v) N- laurylsarcosine sodium salt for 30 min at room temp prior to

centrifugation (10°C, 75,600 x *g*, 2 h). The pellet or outer membrane fraction (OM) was washed in 20 mM Tris-HCl (pH 7.5) to remove excess salts and was once again pelleted by centrifugation (10°C, 75,600 x *g*, 2 h). Finally, the OM pellet was solubilised in 1.5 ml of 1 x loading buffer (50 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 10% [v/v] glycerol, 1% [v/v] β-mercaptoethanol, 12.5 mM EDTA, 0.02% [w/v] bromophenol blue). Undissolved OM material was removed by centrifugation (13,400 x *g*, 20 min) before decanting supernatants into clean Eppendorf tubes and storing at -70°C. It was important to store OMPs at lower temps (e.g. -70°C) as high molecular weight proteins have been found to degrade when stored at -20°C (Davies, 1991a).

2.2.5 Purification of native *Y. ruckeri* flagellin

Native flagellin was purified from a motile *Y. ruckeri* serotype O1 (BA19) strain following an acid disassociation and re-association method outlined by Ibrahim *et al*. (1985). A starter culture, which was grown from a single colony overnight in 10 ml of TSB (23°C, 5 x *g*), was used to inoculate 6 x 1 L volumes (1:1000 dilution) of TSB and grown for a further 18 h (23°C, 5 x *g*). Cells were harvested (4°C, 9,605 x *g*, 40 min) and gently mixed with ice cold saline (0.9% [w/v] NaCl) supplemented with 2 mM PMSF to form a moderately thick suspension. The pH of the suspension was then reduced to pH 2 using 1M HCl and maintained at room temp under constant stirring for 40 min to disassociate flagella. Bacterial cells, now stripped of flagellin, were removed by centrifugation (4°C, 9,605 x *g*, 40 min). The supernatant, containing flagellin as soluble monomers, was centrifuged (10°C, 75,600 x *g*, 2 h) to remove insoluble contaminants before adjusting the pH to 7.5 with 1 M NaOH to reinitiate flagellin polymerization. To precipitate the protein, ammonium sulphate was slowly added to the solution with vigorous stirring to achieve two-thirds saturation (2.67 M) before holding overnight at 4°C. The resulting precipitate (= flagellin protein) was centrifuged (4°C, 48,384 x *g*, 30 min) and dissolved in sterile buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl) prior to dialysing in pre-treated dialysis tubing (Section 2.2.8). Dialysis was initially performed under running tap water for 2 h and then for 18 h at 4°C with constant stirring in 6 L of dH_2O. Purity and identity of native flagellin was confirmed via SDS-PAGE (Section 2.2.10) and Western blotting/immunodetection (Section 2.2.16). Prior to administering to fish (Section 2.4.4), the sample was diluted to the required concentration with sterile

phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO4 [pH 7.4]).

2.2.6 Solubilisation and purification of recombinant *Y. ruckeri* flagellin

Pellets of induced cells (Section 2.3.13.3) were re-suspended in 10 ml of buffer A (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM EDTA, 0.1% [w/v] sodium azide, 2 mM PMSF, 1 mg/ml lysozyme) and stored on ice for 30 min. Cells were lysed by passing through a French pressure cell press (3 repeats at 14,000 psi) with 5 min intervals on ice. Recombinant flagellin (r-flagellin), expressed in inclusion bodies (IBs), was pelleted by centrifugation (10°C, 75,600 x *g*, 1 h) before the supernatant (= soluble fraction) was removed. IBs were washed three times in 10 ml buffer A supplemented with 1% (v/v) Triton X-100 to remove any contaminating material. Excess Triton was removed by washing the pellet twice with buffer A without Triton-X100. IBs were denatured and solubilised in buffer B (50 mM NaCl, 2 mM PMSF, 0.1% [w/v] sodium azide, 8 M urea [pH 7.4]) by freeze-thawing, vortexing and sonication. Any insoluble material was removed by centrifugation (4°C, 75,600 x *g*, 30 min) before dialysing in pre-treated dialysis tubing (below). Dialysis was initially carried out under running tap water for 2 h and then for 18 h at 4°C with constant stirring in 6 L of dH_2O. Any precipitate (= insoluble r-flagellin) which formed was dissolved by changing the pH of the solution to 2 with 1 M HCl while stirring at room temp for 30 min. Insoluble material was removed by centrifugation (4°C, 75,600 x *g*, 1 h) before slowly changing the pH to 7.5 using 1 M NaOH. Purity of r-flagellin was confirmed via SDS-PAGE (Section 2.2.10) and Western blotting (Section 2.2.16). Finally, the solution was diluted with sterile PBS to give the required concentration of r-flagellin before administering to fish (Section 2.4.5).

2.2.7 Purification of soluble r-flagellin using HisTrap™ FF Columns

Y. ruckeri r-flagellin, expressed as soluble protein, was purified by passing through a HisTrap™ FF (IMAC) Column (GE Healthcare, UK) following the manufacturer's instructions. Purity of protein elutes was confirmed by running 10 µg of protein on a 10% (w/v) SDS-PAGE (Section 2.2.10).

2.2.8 Preparation of dialysis tubing

Dialysis tubing (Medicell International ltd, molecular weight cut-off = 14 kDa) was cut into strips of 10 to 15 cm and boiled (10 min) in 2% (w/v) sodium bicarbonate/1

mM EDTA (pH 8.0). Tubing was boiled for a further 10 min in 1 mM EDTA (pH 8.0) before storing in 1 mM EDTA at 4°C for up to 1 week. Prior to use, tubing was washed in dH_2O.

2.2.9 Precipitation of proteins using trichloroacetic acid (TCA) in acetone

Protein samples were precipitated by mixing with 4 x sample volumes of ice cold 10% (w/v) TCA dissolved in acetone. Solutions were stored at -20°C overnight, followed by centrifugation (4°C, 48,384 x *g*, 30 min) to pellet protein. The supernatant was discarded before washing the pellet three times with ice-cold acetone to remove residual TCA. Protein pellets were allowed to air dry in a fume cupboard before re-suspending in the appropriate buffer.

2.2.10 1-Dimensional (1-D) sodium dodecyl sulphate polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

2.2.10.1 Casting SDS-PAGE gels

SDS-PAGE was carried out using a discontinuous buffer system described by Laemmli (1970). After assembling the gel casting apparatus (Mighty Small II, Hoefer), the polyacrylamide resolving gel (10-12% [w/v] polyacrylamide [19:1 acrylamide: bisacrylamide, Melford], 375 mM Tris-HCl [pH 8.8], 0.1% [w/v] SDS, 0.1% [w/v] ammonium persulphate (APS), 0.05% [v/v] *N,N,N',N'*-tetramethyl-ethylenediamine [TEMED]) was poured between the glass plates. The gel solution was overlaid with water saturated n-butanol to ensure a flat surface and allowed to polymerise at room temperature for 30 min. After removing the n-butanol, the stacking gel (4% [w/v] polyacrylamide, 125 mM Tris-HCl [pH 6.8], 0.1% [w/v] SDS, 0.05% [w/v] APS, 0.1% [v/v] TEMED) was cast directly over the resolving gel with an appropriate comb in place to create loading wells. Gels were stored overnight at 4°C.

Once set, combs were removed, and the gel (still within the glass plates) was gently placed within the electrophoresis apparatus. Buffer tanks were then filled with 1 x SDS-PAGE running buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, 0.1% [w/v] SDS) before loading the sample and running the gel (below).

2.2.10.2 *Sample loading and electrophoresis*

Protein samples were mixed (1:1) with 2 x sample loading buffer (100 mM Tris-HCl [pH 6.8], 4% [w/v] SDS, 20% [v/v] glycerol, 2% [v/v] β-mercaptoethanol, 25 mM EDTA, 0.04% [w/v] bromophenol blue). For SDS-PAGE analysis of crude whole cell proteins, 1 ml of bacterial culture was centrifuged (13,400 x *g*, 10 min) before resuspending the bacterial pellet in 200 µl of 1 x sample loading buffer. All protein samples were vigorously vortexed for 5 min before heating to 95°C for 15 min. Any insoluble material was removed by centrifugation (13,400 x *g*, 10 min) before loading 10-20µl (1–20 µg protein) into each well. Electrophoresis was carried out at 150 V with water cooling the system for 1.5 h, or until the loading dye had reached the end of the gel. Molecular weight markers (Table 2.2) were run alongside protein samples as standard. Following electrophoresis, gels were carefully removed from the apparatus/glass plates and stained with Coomassie brilliant blue-R250 (Section 2.2.12) or silver nitrate (Section 2.2.14) to visualize proteins. All gels were scanned with an ImageScanner™ II (GE Healthcare) after staining. If the gel was to be used in a Western blot, staining was omitted and proteins were electrophoretically transferred to a nitrocellulose membrane (Section 2.2.16).

Table 2.2: Proteins used as molecular weight markers and their corresponding molecular weights in kiloDaltons (kDa).

Protein	***Molecular weight (kDa)***
Myosin, porcine	200
β-Galactosidase, from *E. coli*	116
Phosphorylase b, from rabbit muscle	97
Bovine serum albumin	66
Ovalbumin, from chicken egg white	45
Carbonic Anhydrase, from bovine erythrocytes	29

2.2.11 2-Dimensional (2D) SDS-PAGE separation of proteins

2.2.11.1 *Preparation of glass plates and gel casting for 2D SDS-PAGE*

Glass plates used to cast large (25.5 × 19.6 cm, 1.5 mm thick) SDS-PAGE resolving gels (GE Healthcare, UK) were soaked overnight in light detergent (1% [v/v] Decon®) before soaking (2-3 h) in 1 M NaOH to remove any remnants of previous

gels. Glass plates were then thoroughly rinsing in dH_2O before wiping wth 70% (v/v) ethanol and air-dried. If gels were to be attached to gels, 4 ml of Bind-Silane solution (GE Healthcare, UK) was applied over each plate surface and allowed to air dry for 2 h. Gels were wiped with a small amount of 70% (v/v) ethanol before casting the gel. Large 10% (w/v) SDS-PAGE resolving gels were cast in the Ettan DaltSix apparatus system (GE Healthcare, UK) following the manufacturers' recommendations. The composition of 10% (w/v) SDS-PAGE resolving gels remained unchanged (Section 2.2.10.1), although solutions were filtered through 0.22 µm porosity filters (Whatman) prior to the addition of TEMED and APS. Again, water saturated n-butanol was applied over the surface of the gel to ensure a flat surface. Gels were allowed to polymerize overnight at room temperature before removing n-butanol and storing at 4°C with 1 x SDS-PAGE running buffer (Section 2.2.10.1) covering the surface.

2.2.11.2 *First dimension - isoelectric focusing (IEF) of proteins*

After precipitating and pelleting protein (Section 2.2.9), the sample was resuspended in solubilisation buffer (7 M urea, 2 M thiourea, 4% [w/v] 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate [CHAPS]) before centrifugation (13,400 x *g*, 20 min) to remove insoluble material. For a 24 cm IEF Immobiline DryStrip (pH 3-11 NL), 300 µg of protein (not exceeding 100 µl volumes) was mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% [w/v] CHAPS, 2% [v/v] pharmalyte [pH 3-11 NL], 40 mM DTT, 0.002% [w/v] bromophenol blue) to a final volume of 450µl. The sample was then evenly distributed over a clean 24 cm ceramic strip holder before lowering a 24 cm Immobiline DryStrip (pH 3-11 NL) over the sample with the cathodic (+) region facing the pointed end of the holder. Care was taken to ensure no air bubbles formed under the plastic strip. To prevent evaporation, DryStrip cover fluid (GE Healthcare) was applied over the strip before covering with the lid. IEF was performed using an IPGphor II manifold following settings suggested by the manufacturers (Table 2.3). Strips were stored at -70°C overnight or reduced and alkylated (Section 2.2.11.3) before running the second dimension (Section 2.2.11.4).

Table 2.3: IEF conditions and settings for 24 cm strips (pH 3-11 NL)

Voltage (V)	*IPGphor™ II setting*	*Time (hours)*
30	Step and hold	12 (active rehydration)
300	Step and hold	2
1000	Gradient	1
1000	Gradient	2
8000	Step and hold	8

2.2.11.3 *Reduction and alkylation of proteins*

Following IEF, the Immobiline strip was carefully removed and placed in a plastic cylinder containing 10 ml of equilibration buffer (75 mM Tris-HCl [pH 8.8], 6 M urea, 29.3% [v/v] glycerol, 2% [w/v] SDS, 0.002% [w/v] bromophenol blue) supplemented with DTT (10 mg/ml). Reduction was performed for 15 min at room temperature with gentle shaking before alkylating the strip for 15 min in a new cylinder containing 10 ml of equilibration buffer with iodoacetamide (25 mg/ml). Equilibrated strips were then used immediately to run the second dimension (below).

2.2.11.4 *Second dimension – SDS-PAGE separation of proteins*

Following IEF and equilibration, gel strips were placed on top of a pre-cast (Section 2.2.11.1) 10% (w/v) polyacrylamide resolving gel with the anodic (+) end of the strip facing the left hand side and the plastic backing touching the larger glass plate. After ensuring the strip was in contact with the gel, the strip was sealed in place by applying an agarose sealing solution (0.5% [w/v] agarose and 0.01% [w/v] bromophenol blue dissolved in 1 x SDS-PAGE running buffer [Section 2.2.10.1]) which had been heated to 100°C in a heating block. Once the sealing solution had set, gels were carefully lowered into the Ettan™ DALTSix (GE Healthcare) electrophoresis apparatus unit containing 4.3 L of 1 x SDS-PAGE running buffer in the lower (anodic) chamber. Furthermore, the electrophoresis unit had been equilibrated to 10°C using a MultiTemp™ III temperature controller unit (GE Healthcare) while ensuring the main pump cooling system was turned on. Any remaining gel holder slots were filled with blanks before filling the anodic chamber with running buffer up to the required volume. The cathodic chamber was then filled with 3 x SDS-PAGE running buffer before sealing the system with the lid.

Electrophoresis was initially carried out at 25°C at 5 W/gel for 45 min and then 17 W/gel (maximum 100 W) for approximately 5 hours or until the dye front had reached the end of the gel. After completion, gels were removed from the electrophoresis unit and stained with colloidal Coomassie brilliant blue-G250 (Section 2.2.13). All gels were scanned with an ImageScanner™ II (GE Healthcare) after staining.

2.2.12 Coomassie brilliant blue-R250 staining

Polyacrylamide gels were stained in Coomassie brilliant blue solution (50% [v/v] methanol, 10% [v/v] acetic acid, 0.1% [w/v] Coomassie brilliant blue-R250) overnight. The stain was discarded, and gels were repeatedly washed in destain solution (10% [v/v] methanol, 10% [v/v] acetic acid) until the background became clear and protein bands were distinct. Finally, the destain solution was discarded and gels were stored in dH_2O.

2.2.13 Colloidal Coomassie blue-G250 staining

2D SDS-PAGE gels (Section 2.2.10.4) were fixed (30 min) in colloidal fixer (10% [v/v] acetic acid, 40% [v/v] ethanol) while gently shaking. Once fixed, gels were stained (4 days) in colloidal Coomassie blue-G250 stain (8% [w/v] ammonium sulphate, 1% [w/w] phosphoric acid, 0.08% [w/v] Coomassie blue-G250, 20% [v/v] methanol). Gels were then rinsed repeatedly in dH_2O until the background became clear and proteins were distinct. Finally, gels were stored in dH_2O.

2.2.14 Silver staining of protein gels

Following SDS-PAGE (Section 2.2.10.2), the gel was carefully removed from the apparatus and fixed (30 min) in fixer A (50% [v/v] methanol, 10% [v/v] acetic acid), followed by fixing (30 min) in fixer B (5% [v/v] methanol, 7.5% [v/v] acetic acid). The gel was then washed in dH_2O (4 x 10 min) prior to sensitizing (30 min) in DTT (5 µg/ml). Next, proteins were stained (30 min) with 0.1% (w/v) silver nitrate before rinsing in dH_2O (4 x 10 min). The gel was developed in 100 ml of ice-cold 3% (w/v) sodium carbonate (+ 0.05% [v/v] formaldehyde) until protein bands became clear and distinct. The reaction was terminated by the addition of 5 ml 2.3 M citric acid (10 min) prior to washing the gel in dH_2O (4 x 5 min). Finally, the gel was stored in 1% (v/v) acetic acid.

2.2.15 Peptide mass fingerprinting (PMF)

Protein spots were excised from Coomassie stained 2D or 1D SDS-PAGE gels using a manual spot picker and digested with trypsin Gold (Promega, UK) following the "In-Gel" protein digestion protocol. Gel plugs were picked with a spot picker (head diameter = 1.5 mm) before placing in a sterile Eppendorf tube. Plugs were destained with 200 µl of PMF destain buffer (100 mM ammonium bicarbonate, 10% [v/v] acetonitrile) twice at 37^{o}C for 45 min. The wash buffer was removed and gels were de-hydrated in 100% (v/v) acetonitrile for 5 min at room temp. At this stage, gel pieces were reduced in size and had become opaque in colour. Acetonitrile was removed before fully drying the gel pellet in a Speed Vac® for 10-15 min. Dried pellets were then re-hydrated in 10 µl of trypsin digestion buffer (40 mM ammonium bicarbonate, 10% [v/v] acetonitrile) supplemented with trypsin Gold (20 µg/ml) and incubated at room temperature for 1 h. Ensuring the gel pellet had fully re-hydrated (going from opaque to clear), the gel was fully covered in trypsin digestion buffer and digested overnight at 37^{o}C.

Once digested, 150 µl of Millipore water was added to the digest before vortexing the sample at room temperature for 10 min. The liquid fraction was retained in a fresh Eppendorf tube before washing the gel twice in extraction buffer (50% [v/v] acetonitrile, 5% [v/v] trifluoroacetic acid) with vigorous vortexing. Liquid fractions were pulled before concentrating peptides using ZipTips® pipette tips as per the manufacturers' instructions. Finally, 1-2 µl was spotted directly onto a MALDI-TOF target plate and run on an Ettan™ MALDI-TOF (GE Healthcare, UK). Peptide masses were then compared to those in the NCBI sequence database.

2.2.16 Western blotting and immunodetection of *Y. ruckeri* flagellin

2.2.16.1 Western blotting

Proteins separated by SDS-PAGE (Section 2.2.10.2) were transferred to a nitrocellulose membrane (0.2 µm pore size, BioRad) using an electrophoretic method previously described by Towbin *et al.* (1979). Following SDS-PAGE, unstained gels were carefully removed from the glass plates and equilibrated in 1 x Towbin running buffer (25 mM Tris-HCl [pH8.3], 192 mM glycine, 20% [v/v] methanol) for 10 min at room temperature. Two Scotch-brite pads, four pieces of Whatman 3 MM paper and a nitrocellulose membrane (all cut slightly larger than the SDS-PAGE gel) were also pre-wetted in 1 x Towbin buffer. Using the EC140 mini

blot module (Thermo), the gel holder cassette was then set up as follows: one pre-soaked Scotch-brite pad was placed on the back (cathode [+]) followed by two pieces of 3 MM Whatman paper. The SDS-PAGE gel was then placed on to the paper and flooded with running buffer before placing the membrane on top. Ensuring no air bubbles were trapped between the membrane and the gel, another 2 pieces of 3 MM paper and pre-soaked Scotch-brite was placed on top to complete the sandwich. The gel holder was then closed and placed in the blotting chamber so that the cathode side was facing the cathode electrode. The chamber was filled with 1 x Towbin buffer and blotted at a constant voltage of 14 V for 2 h. To ensure proteins had efficiently transferred, membranes were stained with Ponceau S solution (0.5% [w/v] Ponceau S, 5% [v/v] acetic acid) for 5 min and gently rinsed with dH_2O. If protein transfer was successful (indicated by visible protein bands on the membrane), then proteins were hybridized with the appropriate antibody (below).

2.2.16.2 Hybridization and detection of flagellin with a monoclonal antibody (MCA) #15D8

Blotted membranes (above) were briefly washed in 1 x TBST (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% [v/v] Tween 20) before blocking (2 h) in Western blocking buffer (1% [w/v] non-fat skimmed milk powder [Marvel] in 1 x TBST). The membrane was then hybridized (2 h or overnight at 4 $^{\circ}$C) with the anti-flagellin MCA antibody #15D8 (Feng *et al.*, 1990) diluted 1:2000 in Western blocking buffer. Unbound antibody was removed by washing (5 x 5 min) in 1 x TBST prior to hybridizing (45 min) with a secondary antibody (1:10,000 dilution of goat anti-mouse horse radish peroxidase-linked IgG in Western blocking buffer). Any unbound antibody was again removed by washing (5 x 5 min) in 1 x TBST before covering the surface of the membrane in Lumi-Light® substrate (Roche). After placing the membrane between two acetate sheets, the X-ray was exposed (1 to 10 min depending on signal intensity) to an X-ray film and developed (below).

2.2.17 Chemiluminescent development of X-ray films

In accordance with the manufacturer's instructions, developer and fixer solutions (Kodak) were diluted 1:5 in dH_2O before use. The exposed X-ray film was developed (1 to 2 min) in developing solution until a signal was observed, washed briefly in dH_2O, and then fixed (3 to 4 min) in fixer solution. Finally, films were

rinsed in dH_2O and air dried at room temperature. All developing stages were carried out in a dark room under a red safety light.

2.3 DNA methods

2.3.1 Genomic DNA purifications

Genomic DNA was purified from fresh bacterial cultures grown overnight using the QIAGEN DNeasy® blood and tissue kit as per the manufacturers' instructions. Genomic DNA was stored at -20°C until required.

2.3.2 Plasmid DNA purifications

Plasmids from *E. coli* cells were purified via the alkaline lysis method following Birnboim (1983). After growing single colonies in 5 ml LB (+ antibiotic) overnight at 37°C, 1.5 ml of this culture was transferred to a sterile Eppendorf tube and centrifuged (13,400 x *g*, 2 min). Supernatant was removed and the bacterial pellet was thoroughly re-suspended in 100 µl GTE buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA) supplemented with 1 µl RNase (100 µg/ml, Fermentas) before incubating at room temperature for 5 min. 200 µl of cell lysis buffer (1% [w/v] SDS, 200 mM NaOH) was then added, mixed by inversion, and stored on ice for 5 min. After incubating, 150µl of 3 M potassium acetate solution (pH 5.5) was added, mixed, and stored on ice for a further 5 min. The solution was centrifuged (13,400 x *g*, 1 min) to harvest unbroken cells and cellular debris, whereas the supernatant (~ 400 µl) was transferred to a fresh Eppendorf tube before adding an equal volume (1:1) of 95% (v/v) ethanol to precipitate plasmid DNA. After incubating at room temp for 5 min, plasmid DNA was pelleted by centrifugation (13,400 x *g*, 2 min) and washed with 1 ml 70% (v/v) ethanol. DNA was again pelleted by centrifugation (13,400 x *g*, 2 min) before removing the supernatant and drying (5-10 min) at 75°C. Finally, the DNA was re-suspended in sterile dH_2O and stored at -20°C.

2.3.3 Restriction enzyme digests

Cleavage of plasmid (1-2 µg) or genomic DNA (up to 10 µg) with restriction endonucleases was routinely carried out in a reaction (total volume of 20 or 50 µl) containing restriction enzyme(s) (1 unit of enzyme per 1 µg of DNA) and enzyme-specific buffer (1 x from 10 x stock). Reactions were brought to the final volume using sterile dH_2O. All restriction enzymes and buffers used in this study are illustrated in Table 2.4. Digests were incubated for 3 h (plasmid digests) or

overnight (genomic DNA digests) and deactivated by heating (as described by the manufacturers) or by gel purifying DNA fragments (Section 2.3.7) after electrophoresis (Section 2.3.6). The efficiency of the DNA digest was confirmed by running a small amount (5 µl) on a 1% (w/v) agarose gel before excising the band of interest and purifying DNA.

Table 2.4: Restriction enzymes and digestion buffers used in this study. All restriction digests were performed at 37°C.

Enzyme	*Buffers*
*Bam*HI	10 mM Tris-HCl (pH 8.0 at 37°C), 5 mM $MgCl_2$, 100 mM KCl, 0.02% (v/v) Triton X-100 and 0.1 mg/ml BSA.
*Eco*RI	50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 mM NaCl, 0.02% (v/v) Triton X-100 and 0.1 mg/ml BSA.
*Hin*dIII	10 mM Tris-HCl (pH 8.5 at 37°C), 10 mM $MgCl_2$, 100 mM KCl and 0.1 mg/ml BSA.
*Nde*I	50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 mM NaCl and 0.1 mg/ml BSA.
*Xho*I	50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 mM NaCl and 0.1 mg/ml BSA

2.3.4 DNA ligations

Up to 10 µg of digested DNA (Section 2.3.3) was ligated in a total reaction volume of 20µl. A vector to insert molar ratio of 1:3 was used as routine. A typical reaction mix (20 µl) contained 2µl of 10 x ligation buffer (400 mM Tris-HCl [pH 7.8], 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP), 1 µl of T4 DNA ligase (5 units/µl, Fermentas), DNA and dH_2O up to the final volume. Digested DNA was allowed to ligate overnight at 16°C before terminating the reaction by heat inactivation (70°C, 10 min). The efficiency of ligation reactions was confirmed by running a small sample (~5 µl) alongside a digested, non-ligated sample on a 1% (w/v) agarose gel (Section 2.3.6).

2.3.5 Polymerase chain reaction (PCR)

2.3.5.1 Primer design

Primers were designed to be ~20 bp long in order to give an annealing temperature between 50°C and 60°C. The 3'-terminal nucleotides of the primers were designed to contain G or C when possible to enhance primer binding to template DNA. Primers were produced by MWG and upon receipt were re-suspended in sterile dH_2O (final concentration of 100 pm/µl) before storing at -20°C. All primers used in this study are illustrated in Table 2.5.

Table 2.5: Names, sequences and melting temperatures (T_m) of primers used in this study. The *Nde*I restriction site for the FLA-F primer is underlined, as is the *Xho*I restriction site for the FLA-R primer.

Primer	*Sequence (5' → 3')*	*T_m (°C)*	*Reference*
AGPT-F	ATTCAACGGGAAACGTCTTG	54	This study
AGPT-R	ACTGAATCCGGTGAGAATGG	54	-
FLA-F	CGCCATATGGCGGTCATTAACACTAAC	61	-
FLA-R	TACTCGAGACGCAGCAGAGACAACACA	64	-
FLG-L	CCGTTGATGTTAGAGGTGAA	50	-
FLG-R	GGCGGCGCAACGGGTACAGC	62	-
OMPA-F	ATGAAAAAGACAGCTATCGCATT	54	-
OMPA-R	TTAAGCCTGTGGCTGAGTCA	56	-
OMPA-F1	CAAGCGCTGGATCAACTGTA	55	-
OMPA-R1	AACTGAACGCCTTGTGCTTT	56	-
TPNRL17–1	AACAAGCCAGGGATGTAACG	55	Larsen *et al.* (2002)
TPNRL13–2	CAGCAACACCTTCTTCACGA	56	-

2.3.5.2 *PCR reactions*

A standard PCR reaction contained 5 µl 10 x Dream*Taq*™ PCR buffer (Fermentas), 1 µl of each primer (100 pmol/µl), template DNA (up to 100 ng of genomic DNA or 10 ng of plasmid DNA), and 0.2 µl (1.5 units) of *Taq* polymerase (Dream*Taq*™, Fermentas) brought to a final volume of 50 µl with (sterile) Millipure dH_2O. For reactions where the PCR product was used for cloning, conditions were as described except 5 µl of 10 x High Fidelity *Taq* buffer (Fermentas) and 0.2 µl (1.5 units) of Pfu *Taq* polymerase (Fermentas) was added instead of Dream*Taq*™ buffer and *Taq* polymerase. The reaction mixture was pipetted into a 0.2 ml thin walled PCR tube (AxyGen, Inc) and placed in a thermal cycler (Gen Amp® PCR system 2700, Applied Biosystems). A standard PCR cycle consisted of an initial denaturation step of 5 min at 94°C followed by variable cycles of denaturation (30 s at 94°C), annealing (30 s at primer specific temperatures) and extension (1 min at 74°C). A final step at 74°C for 7 min completed primer extension before holding at 10°C. Annealing temperatures were considered to be the T_m – 5°C. Negative controls contained dH_2O in place of template DNA. PCR products were analysed by agarose gel electrophoresis (below) and stored at -20°C until required.

2.3.6 Agarose gel electrophoresis of DNA

DNA was analysed by electrophoretically separating fragments using the RunOne™ Electrophoresis Cell (EmbiTec). A 1% (w/v) agarose solution (effective resolution 0.5 to 10 kb) in 0.5 x TBE (44.5 mM Tris-HCl [pH 8.0], 44.5 mM boric acid, 1 mM EDTA) was prepared by melting the agarose in a microwave oven. After cooling the gel below 60°C, ethidium bromide was added to give a concentration of 0.5 µg/ml. The gel was poured into the gel casting platform and allowed to cool with a comb in place. Care was taken to ensure no trapped air bubbles formed underneath the comb itself or on the surface of the agarose gel. Once solidified, combs were slowly removed before placing the gel within the electrophoresis tank and immersing with 0.5 x TBE. DNA samples were mixed with 6 x loading buffer (60% [v/v] glycerol, 60 mM EDTA, 0.09% [w/v] bromophenol blue, 0.09% [w/v] xylene cyanol; Fermentas) and loaded into the wells of the gel. Electrophoresis was carried out at 100 V for 30 min, or until the loading dye reached the end of the gel front, before visualizing DNA with a UV transilluminator (UVItec ltd). A standard molecular weight marker (λ/*Hin*dIII, Fermentas) was run alongside DNA samples to give detectable fragments of 23130, 9416, 6557, 4361, 2322, 2027 and 564 bp on a 1%

(w/v) agarose gel. DNA gels were photographed under UV light using a UVP gel documentation system.

When separating digested genomic DNA for Southern blotting (Section 2.3.12) a 0.7% (w/v) agarose gel was prepared (as above) using the Gibco BRL Horizontal Gel Electrophoresis Apparatus (Life Technologies) system following the manufacturers' instructions. Electrophoresis was carried out at 25 V for 16 h, or again until the dye front had reached the end of the gel.

2.3.7 Gel purification of DNA

After separating on a 1% (w/v) agarose gel (above), DNA (including plasmids and PCR products) was purified using a GeneJET™ gel extraction kit (Fermentas) following the manufacturer's instructions.

2.3.8 Preparation and transformation of chemically competent *E. coli cells*

2.3.8.1 Preparation of chemically competent E. coli cells

Highly competent *E. coli* cells were prepared following a calcium chloride method outlined by Inoue *et al.* (1990). Single colonies were inoculated into 5 ml of LB (+ 20 mM $MgSO_4$) and grown overnight at room temp (approx. 23°C) before using this initial culture to inoculate (1:50 dilution) a 250 ml LB (+ 20 mM $MgSO_4$) culture. Bacteria was again grown at room temperature to mid exponential phase ($OD_{600} \geq 0.4 \leq 0.6$). Next, cultures were stored on ice for 10 min prior to centrifugation (4°C, 5,403 x *g*, 10 min). Bacterial pellets were then re-suspended in 80 ml of ice-cold transformation buffer (PIPES-HCl [pH 6.7], 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$) and stored on ice (10 min) prior to centrifugation (4°C, 5,403 x *g*, 10 min). Finally, cells were resuspended in 20 ml ice-cold transformation buffer (+ 1.5 ml dimethyl sulphoxide) and again stored on ice (10 min) before dispensing into pre-chilled Eppendorf tubes (200 µl volumes). Aliquots were frozen in liquid nitrogen and stored at -70°C until required for transforming (below).

2.3.8.2 Transformation of competent E. coli cells

Competent *E. coli* cells (above) were transformed using the heat shock method outlined by Sambrook *et al.* (1989). Briefly, aliquots were removed from the -70°C freezer and thawed on ice. Up to 100 ng of plasmid DNA (1-20 µl) was added to the thawed culture and briefly mixed by inversion before again storing on ice for a

further 20 min. Cells were then heat shocked for 45 s at 42°C before immediately placing back on ice for 5 min. Transformed cells were allowed to recover by adding up to 1 ml of pre-warmed LB (without antibiotic) to the mixture and incubating at 37°C (5 x *g*, 30 min). After harvesting cells (2,855 x *g*, 2 min), bacterial pellets were re-suspended in ~200 µl of LB before plating onto pre-warmed (37°C) LA plates (+ antibiotic). Plates were allowed to incubate overnight at 37°C.

2.3.9 Bacterial mutagenesis

2.3.9.1 Minimum inhibitory concentration (MIC) determination for counteractive selection

Antibiotic susceptibility for the *E. coli* strain BW2020767/pRL27 (Table 2.1) and *Y. ruckeri* strains (BA19 and EX5, Table 2.1) were initially tested using the Mast Diagnostics Mastring (M13 and M26) following the manufacturers' instructions. The MIC for antibiotics of potential interest was subject to additional testing using serial dilutions. Briefly, overnight cultures ($OD_{600} \geq 1.0$) were diluted (1:100) in fresh media containing the appropriate antibiotic at various concentrations (0, 2, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml) before growing for a further 24 h. The MIC was considered to be the antibiotic concentration which inhibited or significantly reduced bacterial growth ($OD_{600} \leq 0.05$).

2.3.9.2 Transposon mutagenesis

The pRL27 plasmid encoding kanamycin resistance (Km^R) was transferred from the *E. coli* donor strain (BW2020767) to *Y. ruckeri* recipients following a conjugation method previously described by Larsen *et al.* (2002). Briefly, cultures were grown to mid exponential phase ($OD_{550} \geq 0.7 \leq 0.9$) before mixing *E. coli* with *Y. ruckeri* strains in a ratio of 1:10 (50:500 µl) or 1:1 (200:200 µl). Cells were then collected by filtration using a 0.45-µm porosity membrane (Whatman) and allowed to conjugate overnight on a TSA plate at 28°C or room temperature. After conjugation, cells were re-suspended in TSB and plated, with or without serial dilutions, onto selective TSA plates (+ 50 µg/ml kanamycin, + 5 µg/ml nitrofurantoin) to isolate *Y. ruckeri* transconjugants.

2.3.9.3 *Cataloguing mutants*

Following mutagenesis (above), single colonies were picked aseptically using a sterile toothpick or pipette tip and inoculated into one of the 96 wells of a microtitre plate (Greiner) containing 100 µl of TSB (+ 50 µg/ml kanamycin, + 5µg/ml nitrofurantoin). Mutants were grown overnight at 28°C and stored at -70°C following the addition of 100 µl (1:1) glycerol stock solution (65% [v/v] glycerol, 100 mM $MgSO_4$ [pH 8.0]) to each well.

2.3.9.4 *Plasmid recovery and sequencing*

Genomic DNA was purified (Section 2.3.1) from transconjugants of interest before digesting (Section 2.3.3) with *Bam*HI, a restriction enzyme which does not cut within the Tn-Km^R transposon (Larsen *et al*., 2002). Next, genomic DNA was ligated (Section 2.3.4) and used to transform chemically competent *E. coli* DH5α/λ*pir* strains (Table 2.1) via the heat shock method (Section 2.3.8.1) before plating onto pre-warmed NA plates (+ 50 µg/ml kanamycin) and incubating overnight at 37°C. Resulting colonies were picked and grown in 10 ml of NB overnight (+ 50 µg/ml kanamycin) before purifying the recombinant plasmid (Section 2.3.2) and sequencing with transposon flanking primers TPNRL17–1 and TPNRL13–2.

2.3.10 Screening for non-motile *Y. ruckeri* serotype O1 (BA19) mutants

A diagram outlining the screening and plasmid rescue process for isolating the non-motile serotype O1 (BA19) transconjugant (termed BA19/RL27) is shown in Fig 2.1. Transconjugants were picked and grown in 96 well microtitre plates (Section 2.3.9.3) before screening on motility agar (Section 2.1.4.4) using a 96 well replicator (Greiner). Strains which did not exhibit motility were confirmed by microscopy and by stab inoculating fresh motility plates with a single colony. If a lack of motility was confirmed, then plasmids were recovered for sequencing (Section 2.3.2).

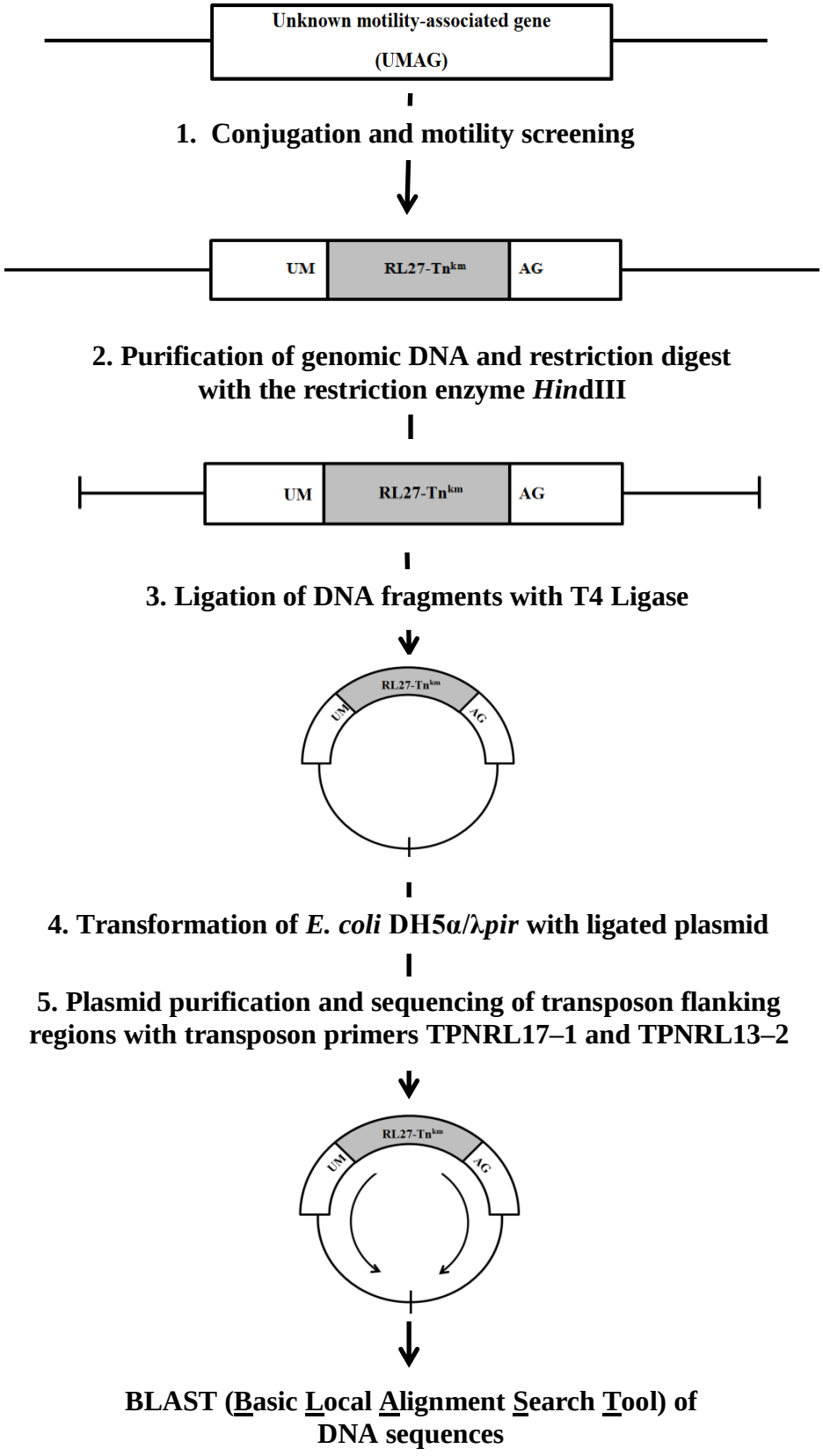


Figure 2.1: Diagram outlining the screening of *Y. ruckeri* serotype O1 (BA19) tranconjugates for a non-motile phenotype and the recovery of plasmid DNA from the genome for sequencing.

2.3.11 Screening for *Y. ruckeri ompA* (*ompA::*Tn-RL27) mutants

EX5 transconjugants grown in 96 well microtitre plates (Section 2.3.9.3) were pooled in groups of 200 before purifying genomic DNA (Section 2.3.1). Transconjugants were screened for a transposon insert within the *ompA* gene (*ompA::*Tn-RL27) using PCR (Fig 2.2). Primer OMPA-F was used in a PCR reaction with primer TPNRL17–1 or TPNRL13–2, whereas reactions with primer OMPA-R were carried out in conjunction with primer TPNRL17–1 or primer TPNRL13–2. Multiple PCRs in this case had to be performed with different primer sets as the Tn-RL27 transposon could have potentially inserted within the *ompA* gene in different orientations. Any resulting PCR products were hybridized with an *ompA* DIG-labelled PCR product using southern blotting (below). PCR products which gave a positive reaction were investigated further by reducing the number of strains in each test group (i.e. 200 to 4 x 50) in an attempt to isolate the transconjugants of interest.

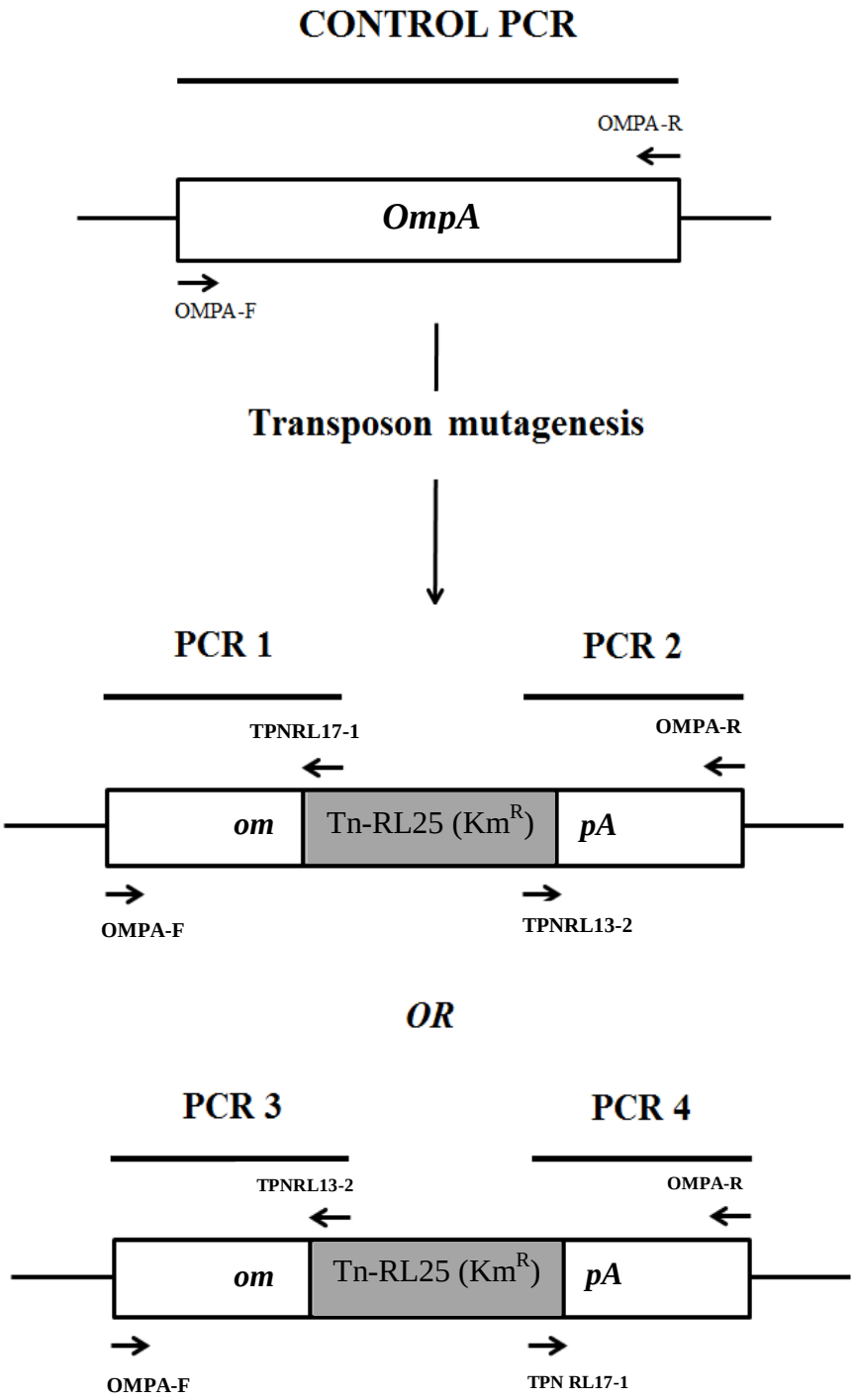


Figure 2.2: Outline of PCR reactions performed to identify a *Y. ruckeri* EX5 (R1) transconjugants which carries a transposon within the *ompA* gene (*ompA*::Tn-RL27).

2.3.12 Southern blotting, hybridization and detection of DNA

2.3.12.1 Preparation of a Digoxigenin (DIG) labeled DNA probes

A 50µl standard PCR reaction was carried out as stated above (Section 2.3.5.2) with the addition of 1 µl 25 mM d UTP-DIG (Roche) to the reaction mixture. The cycle parameters remained the same, albeit with a doubling of the extension time at 72°C to allow the incorporation of DIG-d UTP into the PCR product. To ensure that the incorporation of DIG was successful, original PCR products (without DIG) were run alongside the DIG labeled products. If the incorporation of DIG was successful, the PCR product with DIG-dUTP ran higher in the gel compared to the original PCR products.

2.3.12.2 Southern blotting

Southern blotting was carried out using downward capillary blotting previously performed by Chomczynski and Mackey (1994). Genomic DNA (~10 ug) was digested with *Bam*HI (Section 2.3.3) and separated on a 0.7% (w/v) agarose gel (Section 2.3.6) before being photographed under a UV light. The digested DNA was then depurinated by soaking the gel in 250 mM HCl for 30 min, followed with a brief wash in dH_2O before submerging the gel in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 2 x 20 min. Following another brief wash in water, the gel was placed in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.0]) for 2 x 20 min prior to assembling the blotting stack.

Stacks consisted of ~7 cm of thick paper towels upon which 2 pieces of Whatman 3MM paper, which was pre-soaked in 20 x SSC (1.5 M NaCl, 150 mM sodium citrate [pH 7.0]) and cut to the same size as the gel, was placed on top. A positively charged membrane, which had been rinsed in dH_2O before wetting in 20 x SSC, was placed on top of the Whatman paper. The gel, after removing the loading wells, was then placed upon the membrane before removing any air bubbles by rolling a glass pipette gently over the assembly. To complete the stack, 2 long pieces of 3 MM paper the same width of the gel was placed on top to build a bridge between a buffer tank filled with 20 x SSC. In order to prevent the stack from drying, the assembly was covered in cling film. Blotting took place for at least 8 h or overnight before disassembling the stack and covalently fixing the DNA onto the membrane using a UV crosslinker (UVC-508, Anachem) emitting 120,000 µJ of short-wavelength UV

light. Excess salt was removed by washing the membrane in dH_2O and air-dried at room temperature before hybridization and detection of DNA fragments (bleow).

2.3.12.3 Hybridisation and detection of DNA

DNA blots were firstly pre-hybridised for 1 h in DIG Easy Hyb Buffer (Roche) at 65°C in a rotating roller bottle within a hybridization oven (Hybaid). The DIG-labelled DNA probe was then denatured (100°C, 10 min) before adding up to 25 ng/ml of probe and hybridizing overnight at 65°C. Following hybridization, the membrane was washed once in pre-warmed (65°C) low stringency wash buffer (2 x SSC, 0.1% [w/v] SDS) and then twice in pre-warmed (65°C) high stringency wash buffer (0.2 x SSC, 0.1% [w/v] SDS). Next, the membrane was briefly washed in DIG 1 buffer (100 mM Tris-HCl [pH 8.5], 1 M NaCl, 0.2% [v/v] Tween 20) and then incubated in blocking solution (0.5% [w/v] blocking reagent in DIG 1 buffer) for 1 h at room temperature with gentle shaking. Anti-DIG conjugated alkaline phosphatase (Roche) was diluted (1:20,000) in blocking buffer and incubated with the membrane for 30 min at room temperature. After washing 5 x in DIG 1 buffer, the membrane was equilibrated in detection buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl) for 5 min before evenly spreading 2 ml of the chemiluminescent substrate CDP-Star (Roche) over the surface of the membrane. Finally, the membrane was gently pressed between two sheets of acetate and exposed to an X-ray film for 1 to 20 min (depending on signal intensity) and developed (Section 2.2.17).

2.3.13 Cloning and overexpression of the *Y. ruckeri* flagellin (*fliC*) gene

2.3.13.1 PCR amplification of the Y. ruckeri fliC gene

Specific forward (FLA-F) and reverse (FLA-R) flagellin primers (Table 2.6) were designed using conserved N- and C- terminals of *fliC* from *Y. enterocolitica* (GenBank accession No: ADB96220.1) as a sequence template. This *Y. enterocolitica* strain was chosen due to a protein-protein BLAST (Basic Local Alignment Search Tool) showing 100% identity to 185 amino acid flagellin fragment of *Y. ruckeri* ATCC 29473 (GenBank accession No: ZP_04618012.1). *Y. ruckeri* genomes were shown to possess these primer-specific sequences by performing a DNA BLAST on the sequenced genome of *Y. ruckeri* ATCC 29473. In addition, FLA-F and FLA-R primers included 5' sequences which would allow PCR fragments to contain *Xho*I and *Nde*I restriction sites (underlined in Table 2.6).

Amplification of *Y. ruckeri* flagellin was performed using genomic DNA isolated from the motile Serovar O1 BA19 strain. PCR conditions were as above (Section 2.3.5.2) albeit with an annealing temp at 55°C for the first 5 cycles followed with an annealing temp at 64°C for 25 cycles. The initial reduction in annealing temperature (55°C) allowed the incorporation of *Xho*I and *Nde*I restriction sites within the PCR product.

2.3.13.2 *Cloning of the Y. ruckeri fliC gene*

After running a small amount (5 µl) of the PCR mix on a 1% (w/v) agarose gel (Section 2.3.6) to ensure that the *fliC* gene was successfully amplified, DNA products were digested with *Xho*I and *Nde*I (Section 2.3.3). The pET28-b plasmid was also digested overnight with *Xho*I and *Nde*I. After digesting, PCR products and plasmid DNA were gel purified (Section 2.3.7) and ligated together (Section 2.3.4) to create YRF1 (pET28-b + *fliC*). Chemically competent *E. coli* XL1-Blue cells (Table 2.1), prepared using the calcium chloride method (Section 2.3.8), were transformed with 10 µl of this ligation mixture before plating onto pre-warmed LA (+ 50 µg/ml kanamycin) plates. After incubating at 37°C overnight, any resulting colonies were grown in LB (+ 50 µg/ml kanamycin) before purifying plasmids (Section 2.3.2) and digesting with *Xho*I and *Nde*I to confirm the presence of insert. A positive *E. coli* XL1-Blue transformant which contained the YRF1 construct (termed *E. coli* XL1-Blue/YRF1) was stored at -70°C with glycerol (Section 2.1.3) or used to purify the recombinant plasmid.

2.3.13.3 *Induction and overexpression of Y. ruckeri flagellin*

Purified plasmid YRF1 (Section 2.3.2) was used to transform chemically competent *E. coli* BL21-DE3 strains (Table 2.1) using the heat shock method (Section 2.3.8). Transformants were selected by plating out on LA plates (+ 50 µg/ml kanamycin) and grown overnight at 37°C. Resulting colonies (termed *E. coli* BL21-DE3/YRF1) were inoculated into 10 ml of LB (+ 50 µg/ml kanamycin) and grown for a further 18 h (37°C, 5 x *g*). This starter culture was used to inoculate fresh LB (+ 50 µg/ml kanamycin, 1:100 dilution) and grown (37°C, 5 x *g*) until reaching mid to late log phase ($OD_{600} \geq 0.6$). Gene expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and grown for a further 4 h. Induced cells were harvested (4°C, 9,605 x *g*, 30 min) before purifying

the recombinant protein as IBs (Section 2.2.6) or by passing soluble protein through an IMAC column (Section 2.2.7).

2.4 Fish trials

2.4.1 Ethics statement

All vaccinations and bacterial challenges were carried out in compliance with the Animals (Scientific Procedures) Act 1986 by a UK Home Office license holder (Ref: PIL60/00107) under instruction. Rainbow trout were sedated by immersion in anaesthetic (0.1% [w/v] Ethyl 3-aminobenzoate methane sulphonate salt [MS222]) prior to intraperitoneal (i.p.) injection and monitored for 15 min post-injection to ensure recovery from anaesthesia. Handling and movement of livestock was limited to prevent overstressing. When required, fish were killed by immersion in concentrated MS222. Remaining carcasses were disposed of following university regulations.

2.4.2 Maintenance of fish stocks

Non-vaccinated rainbow trout (average weight = 4 to 5g) were purchased from a commercial fish farm in Scotland. The health status of fish was checked at 48 h after arrival to the aquarium and then every two weeks according to Austin and Austin (1989). Livestock were maintained in continuously aerated, dechlorinated and free flowing freshwater at approx. 12°C in polypropylene tanks. Fish were fed daily to satiation with a commercial pellet diet (Trouw Nutrition, UK).

2.4.3 Preparation of whole cell vaccines

Bacterial whole cell vaccines for fish vaccinations were prepared following Austin *et al.* (2003). Bacterial cells were grown overnight (28°C, 5 x *g*) in 1 L of TSB (+ antibiotic when required) before the addition of formaldehyde to a final concentration of 0.5% (v/v). Cultures were maintained at room temperature for 72 h to allow the inactivation of cells before confirming sterility by spreading 100 µl volumes of culture over 10 TSA plates and incubating for 7 days at 28°C. Bacteria were considered inactive if colonies failed to form following the incubation period. Once sterility was confirmed, 40 ml of culture was centrifuged (4°C, 4,355 x *g*, 20 min) before gently re-suspending the bacterial pellet in 40 ml of sterile PBS. Cell density was determined using a haemocytometer slide (Improved Neubauer Type,

Merck) at a magnification of x1000. Cultures were adjusted, if necessary, with sterile PBS to a final concentration of 5 x 10^8 cells/ml before storing at 4°C.

2.4.4 Dose determination of native *Y. ruckeri* flagellin

Native flagellin purified via the acid disassociation and re-association method was administered to fish (average weight = 5-6 g) by i.p. injection over a range of concentrations (0, 10, 20, 30, 40, 50, 100, 150, 200 and 250 µg/fish) in sterile PBS to a final volume of 100 µl/fish. For each concentration, 5 rainbow trout were vaccinated (N = 5). Fish were monitored over 14 days to determine the highest concentration of native flagellin which did not cause mortality.

2.4.5 Dose determination of recombinant *Y. ruckeri* flagellin

Recombinant flagellin (r-flagellin) purified from inclusion bodies was administered to rainbow trout (average weight = 5 to 6 g) by i.p. injection over a range of concentrations (0, 10, 20, 30, 40, 50, 100, 150 and 200 µg/fish) in sterile PBS to a final volume of 100 µl/fish. For each concentration, 5 fish were vaccinated (N = 5). Fish were monitored for a period of 14 days post-vaccination.

2.4.6 LD_{60} dose determination of *Y. ruckeri* challenge strains YR1 and R1

A lethal dose for 60% of the population (LD_{60}) was calculated by the Probit method of Wardlaw (1985) for strains BA19, YR1, EX5 and R1 (Table 2.1). Thus, groups of rainbow trout (N = 5) were i.p. injected with 100 µl/fish of pathogen suspended in saline ranging from 10^5 to 10^9 cells/ml before recording mortalities over 14 days.

2.4.7 Fish immunizations and bacterial challenges

Rainbow trout (average weight = 5 to 6 g) were randomly chosen from fish stocks and vaccinated via i.p. injection with 100 µl volumes of inactivated whole cell vaccines (5 x 10^7 cells/fish, Section 2.4.3), native flagellin (50 µg/fish, Section 2.2.5) or recombinant flagellin (12, 25 or 50 µg/fish, Section 2.2.6). Controls were i.p. injected with 100 µl volumes of sterile PBS. During the 28 day period between vaccination and challenge, all experimental fish remained within the one tank, but differentiated by various clippings of the caudal fin. Livestock were then separated into different tanks 48 h prior to i.p. challenge with YR1 (9 x 10^6 live cells/fish) or R1 (4.5 x 10^6 live cells/fish) in a total volume of 100 µl saline. The number of cells administered to fish was estimated to be the LD_{60} values for non-vaccinated rainbow

with an average weight of 5 to 6 g (above). Control fish were challenged either with YR1 or R1 (as above) or i.p. injected with sterile PBS. Test subjects were monitored daily after challenge and then more frequently following mortality. After 14 days post-challenge, any remaining fish were killed in concentrated anaesthetic. The layout of initial vaccinations and bacterial challenges undertaken are represented in Table 2.6, whereas the second set of fish trials associated with r-flagellin as a vaccine at various concentrations is illustrated in Table 2.7 and Table 2.8. Data from fish experiments was statistically analysed using methods outlined in Section 2.4.9. Bacteria from dead or moribund fish were reisolated and identified (Section 2.4.8).

Table 2.6: Experimental design of initial vaccinations and bacterial challenges for rainbow trout.

Tank	*No of fish (N)*	*Vaccine type*	*Challenge strain*
A	25	Saline (Control)	Serotype O1[b]
	25	Serotype O1[a]	Serotype O1[b]
B	25	Saline (Control)	EX5[b]
	25	Serotype O1[a]	EX5[b]
C	25	Serotype O1[c] (Non-motile mutant)	Serotype O1[b]
D	25	Serotype O1[c] (Non-motile mutant)	EX5[b]
E	25	EX5[a]	Serotype O1[b]
F	25	EX5[a]	EX5[a]
G	38	Native flagellin (50 µg per fish)	Serotype O1[b]
H	37	Native flagellin (50 µg per fish)	EX5[a]

Abbreviations: Serotype O1[a] = BA19 (WT), Serotype O1[b] = YR1 (WT), EX5[a] = EX5 (WT), EX5[b] = R1 (WT)

Table 2.7: Second phase of fish vaccinations with r-flagellin as a vaccine at different dosages. Bacterial challenge was performed 28 days post-vaccination.

Tank	*No of fish (N)*	*Concentration of r-flagellin (µg per fish)*	*Challenge strain*
A	75	50 (N = 25)	Serotype O1[a]
		25 (N = 25)	-
		12 (N = 25)	-
B	75	50 (N = 25)	EX5[a]
		25 (N = 25)	-
		12 (N = 25)	-
C	25	0 (PBS control)	Serotype O1[a]
D	25	0 (PBS control)	EX5[a]

Abbreviations: Serotype O1[a] =YR1 (WT), EX5[a] = R1 (WT)

Table 2.8: Third phase of fish vaccinations using r-flagellin as a vaccine at different dosages. All fish were maintained within the same tank and were challenged with the *Y. ruckeri* R1 (EX5) strain 14 days post-vaccination.

No of fish (n)	*Concentration of r-flagellin (µg per fish)*
20	0 (PBS control)
20	10
20	25
20	50

2.4.8 Bacteriological examination of infected fish

Bacteria were recovered from dead or moribund fish following challenge. From each test group (including the controls), five fish were examined. Firstly, fish surfaces were washed with 70% (v/v) ethanol before dissecting with a sterile blade. Internal organs (e.g. liver, kidney and spleen) were swabbed before streaking onto a TSA plate and incubating overnight at 28ºC. Once grown, bacteria were streaked out onto

a fresh TSA plate and incubated (28ºC) until single colonies formed. Isolates were identified using bacteriological methods previously outlined in Section 2.1.3.

2.4.9 Statistical analysis

Experimental analysis was calculated as the absolute percentage (%) of fish surviving two weeks post-challenge. In addition, relative percentages (RPS*) of trout survival was calculated after Amend (1981).

$$RPS\ *\ (\%) = \left[1 - \left(\frac{Percent\ mortality\ in\ vaccinated\ group}{Percent\ mortality\ in\ control\ group}\right)\right] \times 100$$

CHAPTER 3: RESULTS

3.1 Bacteriological examination of *Y. ruckeri* isolates

Using bacteriological methods described earlier (Section 2.1.4), all presumptive *Y. ruckeri* isolates were confirmed as belonging to this bacterial species. Furthermore, strains were biotyped depending on their ability to exhibit motility and degrade Tween 20/80 (Table 3.1). Biochemical and morphological phenotypes for all *Y. ruckeri* cultures used in this study were in agreement with Austin *et al.* (2003).

Table 3.1: Selected phenotypes of *Y. ruckeri* strains used in this study.

Strain	*Sorbitol*	*Motility*	*Tween 20/80*	*Biotype*
Serotype O1				
BA19	-	+	+	1
YR1	-	+	+	1
EX5				
EX5	-	-	-	2
R1	-	-	-	2
Serotype O2				
O2BA2	+	+	+	2
Serotype O3				
O3BA3	+	+	+	2
Serotype O4				
O4BA4	+	+	+	2

3.2 Pathogenicity of *Y. ruckeri* strains towards naïve rainbow trout

It was possible to estimate the LD_{60} values of certain *Y. ruckeri* strains by i.p. challenging naïve (= non-vaccinated) rainbow trout with increasing amounts of bacteria (Section 2.4.6). It was estimated that LD_{60} values for the two *Y. ruckeri* EX5 strains used in this study (termed EX5 and EX5 [R1]) was approx. 4×10^5 cells/fish, although this value was higher for a serotype O1 (termed YR1) isolate (8×10^5 cells/fish). The higher LD_{60} value of the serotype O1 (YR1) strain, when compared to the EX5 isolates (EX5 or EX5 [R1]), suggests that this serotype O1 strain is not as virulent.

The second serotype O1 *Y. ruckeri* strain, termed BA19, was the strain used in this study to create the non-motile mutant (BA19/Tn-RL27). Indeed, this BA19 strain is considered to be the isolate used to manufacture the original (= monovalent) ERM vaccine (Prof. B. Ausin, University of Stirling, *personal communication*). However, the BA19 strain was considered to be avirulent since injecting naïve fish with this isolate over a range of concentrations (i.e. 1 x 10^5, 10^6, 10^7, 10^8, 10^9 cells/ml; N = 5 x 5) did not result in any mortalities 14 days post-challenge (100% survival).

3.3 Constructing a non-motile *Y. ruckeri* strain for vaccinations

3.3.1 Optimizing mutagenesis

Random genomic transposon mutagenesis was employed in this study to convert a motile (= serotype O1) *Y. ruckeri* isolate to a non-motile (= EX5) phenotype. Two types of transposon delivery vectors were available for use in this work. The first, termed pRL27, carries a mini Tn*5* (Tn-RL27) transposon and a hyperactive Tn*5* transposase (Larsen *et al.*, 2002). The role of the transposase enzyme is to bind to the ends of a transposon, excise it, then randomly integrate the transposon into the host genome in a "cut-and-paste" manner. Transposase encoded within the pRL27 construct is expressed from the *tetA* promoter and is considered to be 1000 times more active than the wild-type transposase (Larsen *et al.*, 2002). This Tn-RL27 transposon also carries a gene which confers resistance towards kanamycin. In addition, the transposon contains the origin of replication from the plasmid R6K, thereby allowing the insertion site to be cloned via plasmid rescue. Flanking regions can then be sequenced to determine which gene(s) the transposon has potentially disrupted. This process is generally outlined in Fig 2.1. The second transposon delivery vector (termed pLM1) described by Fox *et al.* (2008) is essentially identical to pRL27, albeit carrying the gene required for gentamicin resistance (Gm^R) as opposed to the gene encoding kanamycin resistance (Km^R).

Although both transposon vectors (pRL27 or pLM1) could be administered to bacteria via electrotransformation, bacterial conjugation is generally preferred (Larsen *et al.*, 2002, Fox *et al.*, 2008, Evenhuis *et al.*, 2009). This involves mixing the *E. coli* donor strain (carrying the pRL27 or pLM1 vectors) with the host strain (e.g. *Y. ruckeri*) before filtering bacteria onto a membrane and allowing membrane-bound bacteria to conjugate overnight on media plates (e.g. TSA). Transconjugants (i.e. host strains which carry the Tn-R27 [Km^R] construct) can then be recovered by

plating the conjugation mix out onto medium containing antibiotics which select against the donor and untransformed host strains.

For conjugation to be applied in this study, *Y. ruckeri* isolates would have to be sensitive to kanamycin or gentamicin to allow for mutant selection. Whereas both the serotype O1 isolate (BA19) and EX5 (R1) isolates failed to grow on TSA plates containing either kanamycin (50 µg/ml) or gentamicin (10 µg/ml) after 48 h at 28°C. Therefore, either one of the transposon vectors could be used for mutagenesis. However, the pRL27 plasmid was eventually chosen for use in this study because it has been previously applied by Evenhuis *et al.* (2009) to screen for non-motile *Y. ruckeri* isolates.

While kanamycin could be used to select for transformed *Y. ruckeri* strains, it could not be used to remove the *E. coli* donor strain (in this case *E. coli* BW20767/pRL27) because it is resistant to this antibiotic. The antibiotic employed by Evenhuis *et al.* (2009) to remove the *E. coli* donor strain after mating was not stated.

A second antibiotic was therefore required to counteract the growth of the *E. coli* donor strain (BW20767/pRL27). Hence, the *Y. ruckeri* serotype O1 (BA19) and EX5 (R1) strain, along with the *E. coli* BW20767/pRL27 isolate, were tested against a range of antibiotics. Although this part of the study initially focused on creating a non-motile mutant from a motile parent strain, the EX5 (R1) isolate was included in testing antibiotic susceptibility as this strain was required for transposon mutagenesis at a later stage. Antibiotic susceptibility test patterns for each of the three strains tested are shown in the appendix (Table 5.2). From these results it was clear that the *Y. ruckeri* and *E. coli* donor strain shared a great deal of similarity in antibiotic resistance patterns. It is worth noting that the *Y. ruckeri* serotype O1 (BA19) strain, obtained from a lyophilised culture since 1983, exhibited broad levels of antibiotic resistance (appendix; Table 5.2). This indicates that *Y. ruckeri* strains in 1983 had already developed a strong resistance to most of the conventional antibiotics (e.g. erythromycin) which were used to treat ERM.

Y. ruckeri strains (BA19 and R1) exhibited higher levels of resistance towards nitrofurantoin compared to the *E. coli* donor strain (BW20767/pRL27). The MIC of this antibiotic for the *Y. ruckeri* isolates was 10 µg/ml, but significantly lower (2

µg/ml) for the *E. coli* donor strain (appendix; Fig 5.3). Transconjugants could therefore be obtained after mating by plating out onto TSA or NA plates supplemented with 50µg/ml kanamycin and 5µg/ml nitrofurantoin. In addition, culturing at 28°C, as opposed to 36°C, also significantly hindered *E. coli* growth. Hence, kanamycin, nitrofurantoin and lower incubation temperatures (e.g. 28°C) could be used to select for *Y. ruckeri* Tn-RL27 Km^R transconjugants (Fig 3.1).

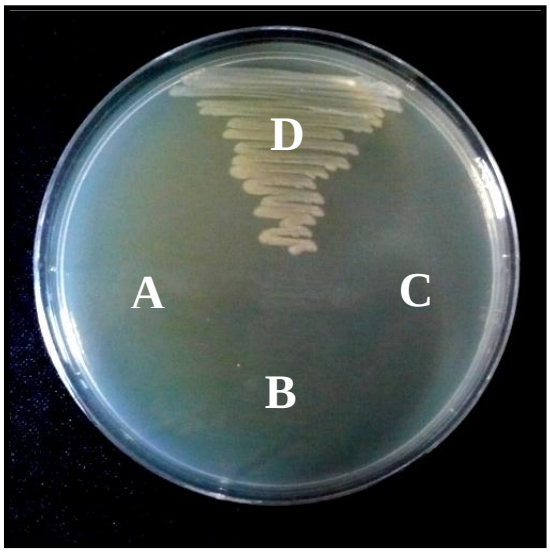


Figure 3.1: Use of temperature and antibiotics to select for *Y. ruckeri* Tn-RL27 (Km^R) transconjugants after mating *Y. ruckeri* cells with the *E. coli* donor strain (BW20767/pRL27). Single colonies were streaked out onto nutrient agar (NA) supplemented with kanamycin (50 µg/ml) and nitrofurantoin (5 µg/ml). Plates were incubated for 48 h at 28ºC. **A:** Wild-type (WT) serotype O1 (BA19) *Y. ruckeri* strain. **B:** WT EX5 *Y. ruckeri* (R1) isolate. **C:** *E. coli* BW20767/pRL27 donor strain. **D:** *Y. ruckeri*/Tn-RL27 (Km^R) transconjugant (BA19/Tn-RL27).

The conjugation efficiency between the *E. coli* donor strain with that of the *Y. ruckeri* BA19 or EX5 (R1) isolate was investigated. A standardized conjugation (Section 2.3.9.2) resulted in a transformation efficiency of $1.2 \pm 0.5 \times 10^7$ cells/ml, approx. equating to $1.2 \pm 0.5 \times 10^8$ transconjugants from one mating experiment. The number of transconjugants was not significantly different between the *E. coli* BW20767/pRL27 and *Y. ruckeri* BA19 or R1 strain. This rate of mutagenesis was

considered to be adequate for the purposes of this study. Moreover, contamination with other bacteria (e.g. *E. coli*) was minimal since 20 randomly picked colonies from both transformations were all confirmed to be *Y. ruckeri* using conventional bacteriological techniques (Section 2.1.4).

In addition to ensuring that the transformation efficiency was adequate, the presence of the kanamycin resistance gene (Km^R) and the random integration of the transposon within the genome had to be confirmed. Firstly, a PCR reaction using the kanamycin forward (AGPT-F) and reverse (AGPT-R) primers was performed to amplify the region of DNA containing the kanamycin resistance gene using genomic DNA from 7 randomly chosen transconjugants as templates (Fig 3.2; A). Four of these tranconjugants were from a mating experiment using the *E. coli* donor and *Y. ruckeri* BA19 strain (generically termed BA19/Tn-RL27 1 to 4). The last 3 transconjugants were sourced from a mating experiment using the *E. coli* donor and *Y. ruckeri* EX5 (R1) isolate (termed R1/Tn-RL27 1 to 3). It was clear from this experiment that *Y. ruckeri* transconjugants obtained the Km^R gene via conjugation since all 7 PCR reactions generated a detectable PCR product, whereas reactions using genomic DNA from the WT parent strains did not (Fig 3.2; A).

To determine if the transposon insertions within the genome of these mutants were random, genomic DNA was digested with *Bam*HI and separated on a 0.7% (w/v) agarose gel (Fig 3.2; B). *Bam*HI was chosen as this restriction enzyme does not cut within the kanamycin gene or indeed the transposon itself (Larsen *et al.*, 2002). Digested genomic DNA was then blotted to a membrane before hybridizing with a DIG-labelled Km^R probe (Fig 3.2; C). Should transposons all integrate within the same region of host genomic DNA then hybridized bands between different transconjugants would have all been the same size. This is clearly not the case for hybridized bands visible in Fig 3.2 (C), thus indicative of random transposon insertions (more specifically the Km^R gene) within the genome.

At this stage it was clear that the conjugation system described by Larsen *et al.* (2002) could be successfully used to create *Y. ruckeri* transconjugants.

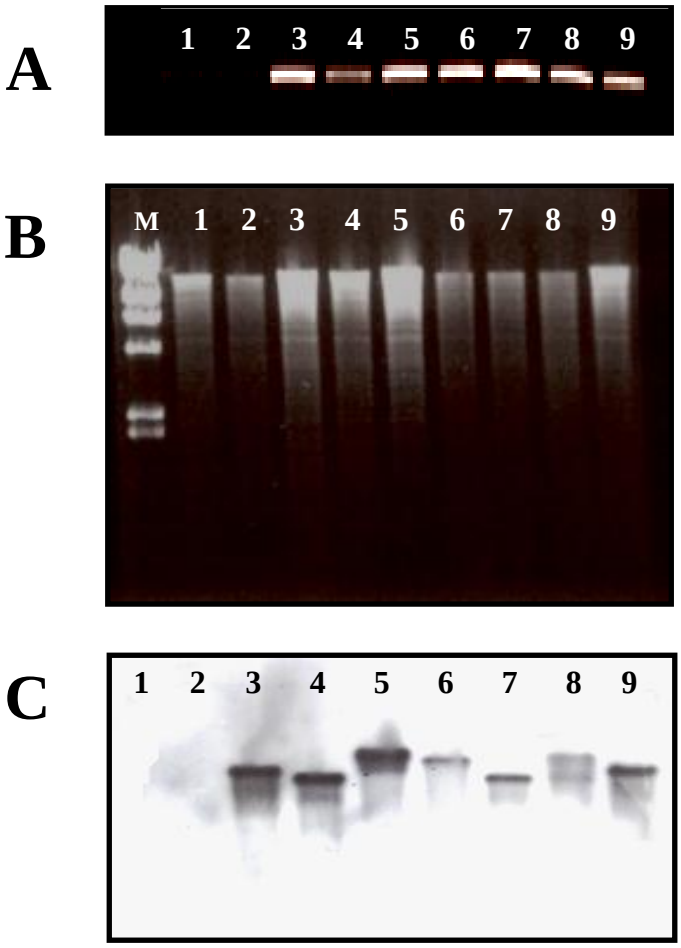


Figure 3.2: Detection of the Tn-RL27 Km^R (*aph*) gene within the genomes of *Y. ruckeri* transconjugants. **A:** PCR amplification of the Tn-RL27 Km^R gene from bacterial genomes using the primers AGPT-F and AGPT-R. **B:** *Bam*HI digestion of genomic DNA separated on a 0.7% (w/v) agarose gel; **C:** Detection of Tn-RL27 Km^R genes using a DIG-labeled Tn-RL27 Km^R PCR product. **M:** Molecular weight marker (λ *Hin*dIII; Fermentas**). Lane 1:** WT serotype O1 (BA19). **Lane 2:** WT EX5 (R1). **Lanes 3-6:** Serotype O1 (BA19) transconjugants (BA19/Tn-RL27 1 to 4). **Lanes 7-9:** EX5 (R1) transconjugants (R1/Tn-RL27 1 to 3).

3.4 Screening for a non-motile BA19 mutant

Matings were performed between the WT *Y. ruckeri* serotype O1 (BA19) and the *E. coli* donor strain (BW20767/pRL27). In total, 4000 *Y. ruckeri* serotype O1 (BA19) transconjugants were randomly picked and grown overnight at 28ºC in individual wells of 96-well microtitre plates containing 200 µl of TSB (+ 50 µg/ml kanamycin, + 5 µg/ml nitrofurantoin). Using a 96-pin microtitre replicator, *Y. ruckeri* transconjugants were stab inoculated onto semi-solid motility media (an example is shown in the appendix; Fig 5.4) and incubated overnight at 28ºC. Each microtitre plate had glycerol added to each well and was stored at -70ºC.

After screening 4000 mutants on motility media, 3 mutants (B2A12, C4C12, and C4F11) were seen to be potentially non-motile. Fresh cultures of all 3 strains were purified from glycerol stocks and again stab inoculated onto motility media. Two strains (B2A12 and C4C12) showed some signs of motility, whereas the third (C4F11) did not. The non-motile phenotype of the C4F11 transconjugant, which was termed BA19/Tn-RL27 for simplicity, can be observed in Fig 3.3.

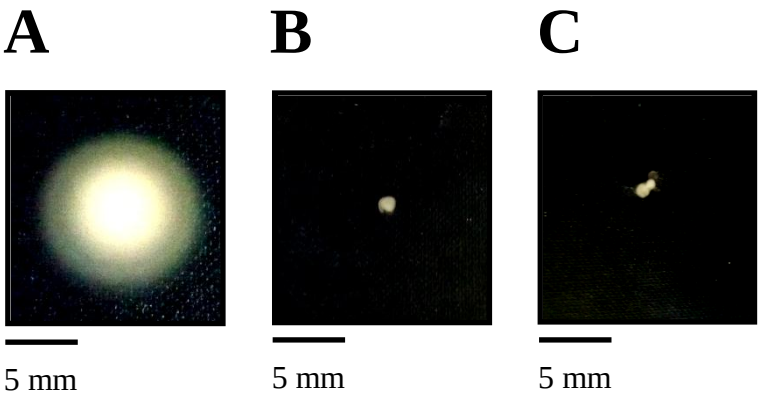


Figure 3.3: Flagellin-mediated motility of *Y. ruckeri* isolates. Single bacterial colonies were stab inoculated onto motility plates (Columbia broth + 0.3% [w/v] agar) and incubated overnight at 28°C. Strains were considered motile if an outgrowth or halo formed from the point of inoculation after incubation. Whereas the WT serotype O1 (BA19) strain is motile (**A**), the WT EX5 (R1) (**B**) and serotype O1 transconjugant (BA19/Tn-RL27) (**C**) are non-motile.

Although the serotype O1 (BA19/Tn-RL27) transconjugant is clearly non-motile, it does not necessarily imply that the flagellin monomer is not produced by this strain. To determine if this protein is expressed by this transposon mutant, whole-cell proteins (WCPs) from both the serotype O1 (BA19) and BA19/Tn-RL27 transconjugant were separated electrophoretically on a 10 % (w/v) SDS-PAGE gel (Fig 3.4; A) before blotting proteins to a nitrocellulose membrane and hybridizing with a monoclonal anti-flagellin (#15D8) antibody (Fig 3.4; B). In addition, WCPs from the non-motile EX5 isolate were run alongside these samples. Proteins in lanes 4, 5 and 6 of Fig 3.4 will be the topic of discussion at a later stage. It is clear from this result that the serotype O1 (BA19) strain is producing a flagellin monomer (approx. 45 kDa) as it is recognised by the anti-flagellin antibody. On the other hand, both the EX5 and non-motile BA19/Tn-RL27 transconjugant are not producing detectable flagellin. Thus, it was clear that the BA19/RL27 mutant was non motile and unable to produce flagellin under growth conditions used to cultivate this strain (i.e. 28ºC in TSB for 48 h at 250 rpm). Like the EX5 strain and the non-motile mutant isolated by Evenhuis *et al.* (2009), the BA19/RL27 transconjugant was unable to produce and/or excrete an extracellular lipase (appendix; Fig 5.5).

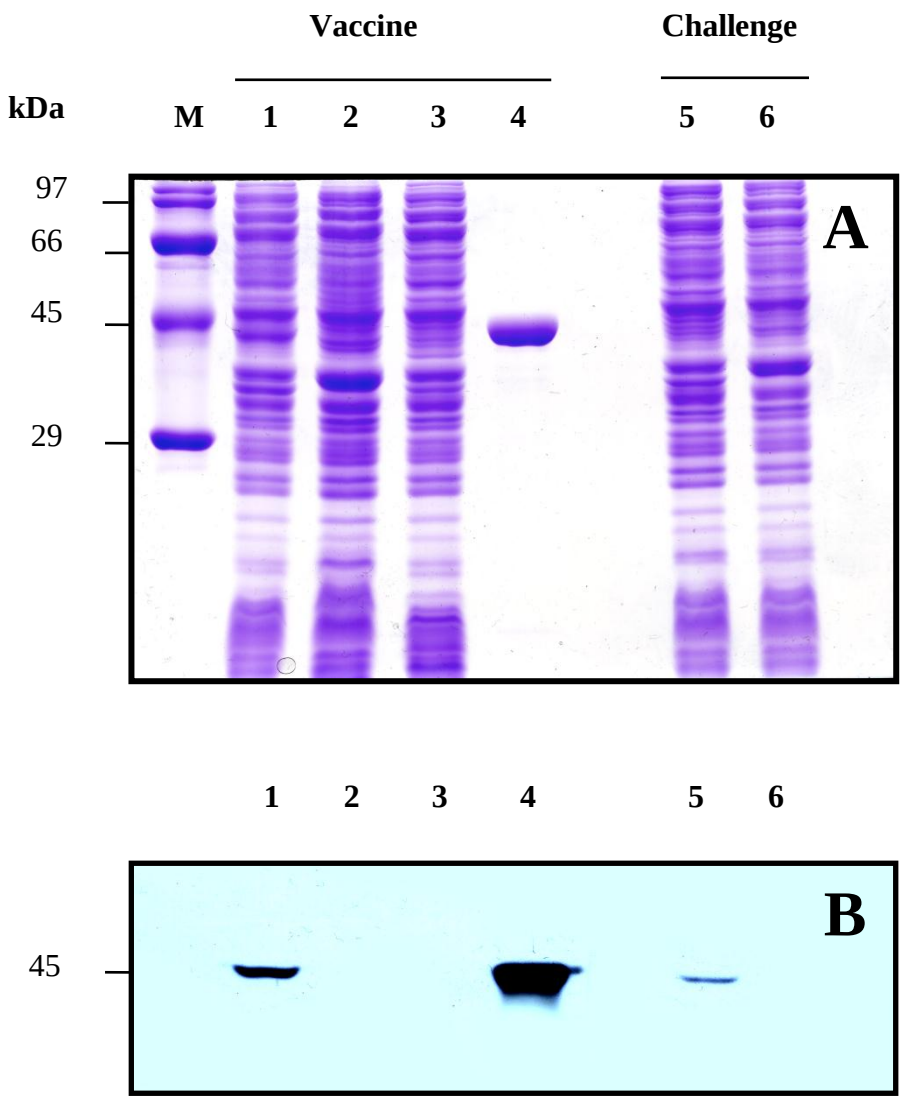


Fig 3.4: Immunodetection of flagellin within vaccines and challenge strains. **A:** Proteins fractionated on a 10% (w/v) SDS-PAGE gel. **B:** Immunodetection of flagellin after blotting proteins to a membrane and hybridizing with the anti-flagellin monoclonal antibody #15D8. **M:** Molecular weight marker (29 to 200 kDa; Sigma). **Lanes 1:** Vaccines used to immunize naïve rainbow trout. **Lanes 5-6:** Whole cell proteins (WCPs) of bacterial strains used for challenges. **Lane 1:** WCPs of a serotype O1 strain (BA19). **Lane 2:** WCPs of the EX5 strain. **Lane 3:** WCPs of the non-motile serotype O1 transconjugant mutant (BA19/RL27). **Lane 4:** Native flagellin (5 µg) purified from the serotype O1 (BA19) isolate via the acid disassociation and re-association method described by Ibrahim *et al.* (1985). **Lane 5:** WCPs of a serotype O1 strain (YR1). **Lane 6:** WCPs of an EX5 (R1) strain. See text for details.

The next stage was to determine the location of the inserted transposon within the bacterial chromosome. For this to be achievable, the transposon and flanking DNA was recovered from the BA19/Tn-RL27 genome in the form of a plasmid for replication and sequencing. A diagrammatic representation of plasmid recovery can be seen in the Materials & Methods (Fig 2.2). Briefly, genomic DNA was purified from the BA19/Tn-RL27 mutant and digested with *Bam*HI. DNA fragments were then ligated with T4 ligase to create synthetic plasmids before using this ligation mixture to transform chemically competent *E. coli* DH5α/λ*pir* bacteria. The transformation mixture was then plated out onto LA (+ 50 µg/ml kanamycin) and incubated at 37˚C for 48 h. Resulting colonies were grown in LB (+ 50 µg/ml kanamycin) before purifying the recovered plasmid. Flanking regions (e.g. genomic DNA sequences to the left and right hand side of the transposon) were sequenced at least twice using the TPNRL17–1 or TPNRL13–2 primer.

BLAST analysis of genomic DNA sequences flanking the transposon showed that the transposon had inserted within the *flhA* gene of *Y. ruckeri* (Fig 3.5), a region of DNA which is believed to encode a protein involved in the flagellar secretion apparatus (Chevance and Hughes, 2008). Annotated DNA sequences regarding this transposon insert are included in the appendix (Fig 5.6). The *flhA* gene of *Y. ruckeri* was also disrupted by Evenhuis *et al.* (2009) using the same transposon mutagenesis method. This would suggest that this particular region is a so-called “hot-spot” for transposon integration. Although the gene encoding flagellin has not been disrupted, the BA19/Tn-RL27 transconjugant is not producing detectable flagellin (Fig 3.4; Lane 3) and can therefore be used in fish vaccination studies.

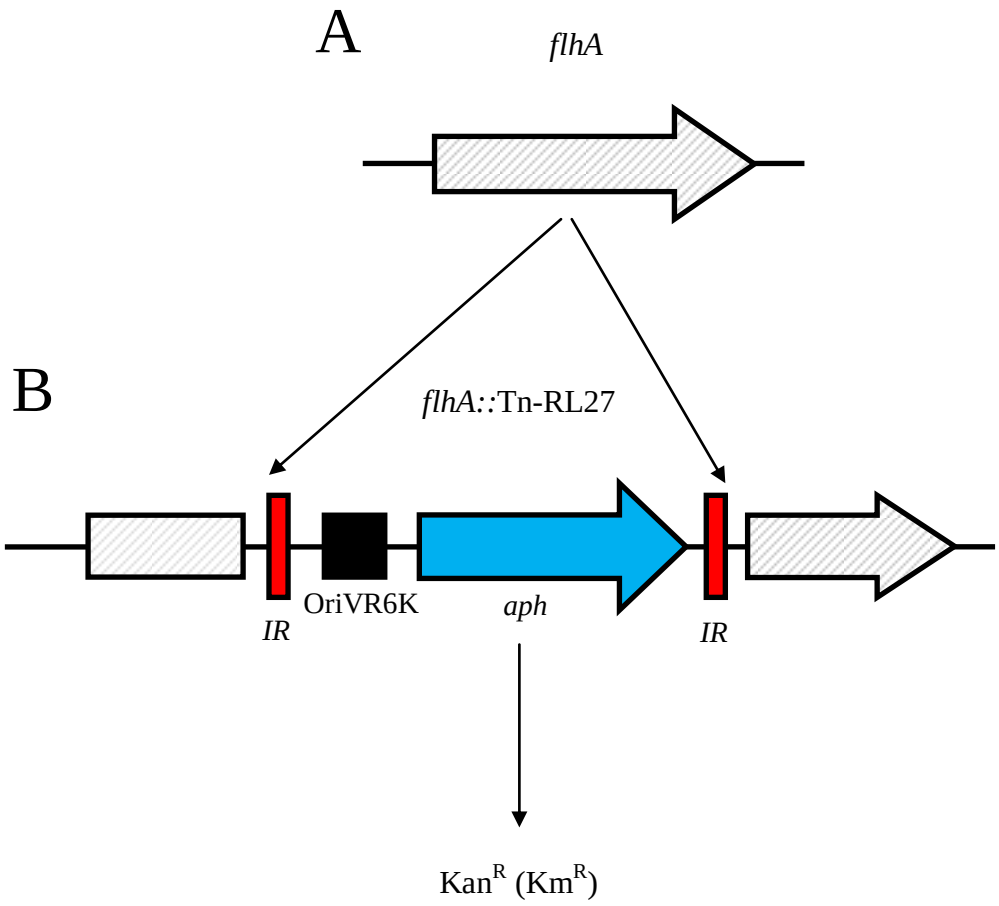


Fig 3.5: Diagrammatic representation of the Tn-RL27 transposon insertion within the *flhA* gene of the serotype O1 (BA19/Tn-RL27) *Y. ruckeri* transconjugant. The WT serotype O1 (BA19) *flhA* site is shown (**A**), whereas the insertion of the Tn-RL27 transposon in the BA19/Tn-RL27 site is also depicted (**B**).

3.5 Flagellin as a component of a whole-cell *Y. ruckeri* vaccine

The protective role of flagellin as a component of a whole-cell vaccine was investigated. Both the presence and absence of flagellin within the formalin-inactivated whole-cell vaccine preparations was confirmed by Western blotting and immunodetection using an anti-flagellin antibody (Fig 3.4) before carrying out immunizations. Similarly, the presence or absence of flagellin in each of the challenge strains was also confirmed prior to challenging fish (Fig 3.4).

All naïve rainbow trout (average weight = 5 to 6 g) mock-vaccinated and/or mock-challenged by intraperitoneal (i.p.) injection with sterile PBS survived (Relative

Percent Survival [RPS] = 100%). This shows that any subsequent mortality in this study was not due to the stress of i.p. injection, handling or submersion in anaesthetic. However, only 32 and 48% of all mock-vaccinated fish survived after challenging with a serotype O1 (YR1) (8 x 10^5 cells/ fish) and EX5 (R1) *Y. ruckeri* strain (4 x 10^5 cells/ fish) respectively. This confirms the pathogenicity of bacterial isolates towards non-vaccinated rainbow trout.

Survival rates 14 days post-challenge for trout i.p. vaccinated with a motile, flagellin-producing *Y. ruckeri* serotype O1 (BA19) strain (1 x 10^8 inactivated cells/fish) is shown in Fig 3.6. Whereas serotype O1 (BA19) vaccinated fish were moderately protected against challenge with a serotype O1 (YR1) isolate (total survival = 76%; RPS = 65%), vaccinates were less protected against the EX5 (R1) strain (total survival = 60%; RPS = 23%). On the other hand, vaccinating fish with the non-motile flagellin-devoid serotype O1 transconjugant (BA19/Tn-RL27) resulted in a reduction in the level of protection against challenge with the serotype O1 (YR1) strain (total survival = 60%; RPS = 41%). This suggests that flagellin is not the only immunostimulatory molecule in the vaccine preparation. However, this reduction in protection was not specific for challenge against a flagellin-producing strain as survival rates for fish vaccinated with the serotype O1 BA19/Tn-RL27 transconjugant was also reduced when challenged with the EX5 (R1) strain (total survival = 46%; RPS = 0%).

It should be noted that, for ethical reasons, numbers of fish were kept to a minimum in each group (N = 25). As a result, the standard error rates are high. When taking this into consideration, total survival values are not statistically significant between survival rates of fish vaccinated with a flagellin producing (e.g. serotype O1 [BA19]) or flagellin-devoid (e.g. serotype O1 [BA19/Tn-RL27]) mutant strain ($P < 0.05$). This indicates that the presence or absence of flagellin does not have a profound effect on the levels of protection towards bacterial challenge, suggesting that *Y. ruckeri* flagellin is not the main immunogenic/immunostimulatory molecule involved in eliciting an immunoprotective response.

Vaccinating with inactivated whole cells of the non-motile, flagellin-devoid *Y. ruckeri* EX5 strain was also included in these fish trials (Fig 3.6). Vaccinating with this strain (1 x 10^8 inactivated cells/fish) gave excellent levels of protection (total

survival = 96%; RPS = 95%) against challenge with the serotype O1 (YR1) strain. This level of protection is significantly higher than that obtainable using the serotype O1 (BA19) or non-motile BA19/Tn-RL27 mutant as a vaccine. Thus, flagellin is clearly not required for conferring a protective immune response against ERM in rainbow trout. Furthermore, EX5 must harbour molecules or epitopes that enhance the protective reaction against serotype O1 (YR1) challenge.

Interestingly, fish vaccinated with the EX5 strain, although strongly protected against challenge with the serotype O1 (YR1) strain, were not protected to the same extent after challenging with the EX5 (R1) strain (72% survival; RPS = 42%). This is difficult to explain, particularly since both the EX5 strains are very similar in terms of biochemistry and physiology. One explanation is that the EX5 isolate is more capable of resisting clearing by the immune system, a feature which is associated with *Y. ruckeri* pathogenicity (see discussion). However, this would require further investigation.

Overall, this study shows that flagellin, or at least flagellin within the whole cell vaccine preparations, is not required to protect against a motile *Y. ruckeri* isolate (e.g. YR1). Although it is possible that flagellin may have a non-specific role in protection in a serotype O1 vaccine, it is not an essential component in developing protection. Results therefore suggest that other molecules, possibly those presented on the surface of EX5 cells, can sufficiently protect against challenge with a serotype O1 (YR1) and, to some extent, the non-motile (e.g. EX5 [R1]) *Y. ruckeri* strain.

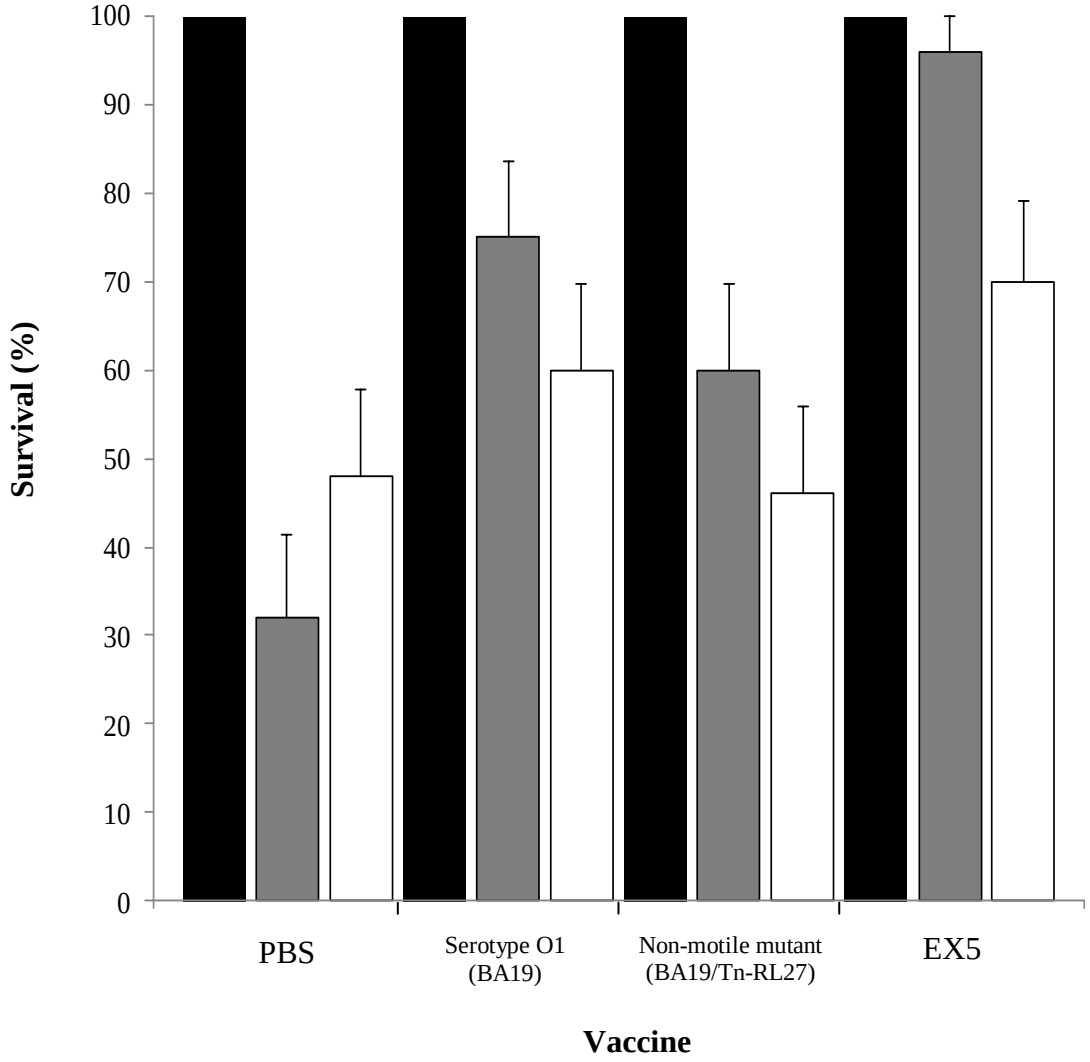


Fig 3.6: Use of formalin-inactivated whole-cell vaccines to protect rainbow trout against challenge with a serotype O1 (YR1) (▩) or EX5 (R1) (□) *Y. ruckeri* strain. Control fish were mock-vaccinated/challenged with PBS (■). Survival rates 14 days post-challenge are given. Standard error values, where applicable, are shown for each group (N = 25).

3.6 Purity of native *Y. ruckeri* flagellin

In addition to investigating the role of flagellin in protecting against ERM as part of a whole-cell vaccine, the use of flagellin as a purified, sub-unit vaccine was also explored. Native flagellin was obtained from live serotype O1 (BA19) *Y. ruckeri* cells following the acid disassociation and re-association method outlined by

Ibrahim *et al.* (1985). Initial attempts were made to acquire flagellin from cultures grown in M9 minimal as this was originally thought to prevent contamination from protein-rich broth (Ibrahim *et al.*, 1985). However, bacterial growth was poor (OD_{600} $\leq$ 0.4), resulting in a low yield of flagellin ($\leq$ 166 µg/L). On the other hand, when grown overnight in 6 x 1 L of TSB, 5 mg of flagellin (approx. 833 µg/L) could be recovered without any apparent contamination with media proteins. No flagellin could be obtained when using the *Y. ruckeri* EX5 (R1) culture as a source of the protein.

After purifying protein using a method described by Ibrahim *et al*. (1985), 5 µg was fractionated on a 10% (w/v) SDS-PAGE gel (Fig 3.4; A: Lane 4). A protein band approximately 45 kDa in weight was clearly visible after staining with Coomassie brilliant blue-R250. No other protein bands were observed. Western blotting and immunodetection with the anti-flagellin antibody (#15D8) confirmed that this 45 kDa protein was flagellin (Fig 3.4; B: Lane 4). Given that only one protein band was observable, this flagellin preparation was considered to be of a sufficient purity for testing protein toxicity for fish (below) and for use as a sub-unit vaccine (Section 3.8).

3.7 Toxicity of native *Y. ruckeri* flagellin to rainbow trout

Native flagellin was intraperitoneally (i.p.) administered to naïve rainbow trout (average weight = 5 to 6 g) at various amounts (0, 10, 20, 30, 40, 50, 100, 150, 200 and 250 µg/ fish) to determine what effect this may have for health. Groups of five fish were used to test each concentration of native flagellin. Antigen was administered in a total volume of 100 µl PBS. Prior to injection, fish were anesthetised. Livestock was then observed for 3 days post-injection, after which any remaining fish were killed by overdosing with anaesthetic as described in Section 2.4.1.

Results from this experiment are shown in Fig 3.7. It was evident that concentrations up to 50 µg of native flagellin/fish did not have a detrimental effect on health. Post-mortem examination of trout showed no signs of disease, nor did they show any physiological changes either externally (e.g. skin, eyes, mouth) or internally (e.g. liver, intestine or spleen). Conversely, fish administered with higher concentrations of the native preparation died. In fact, fish did not fully recover from the anaesthesia

administered for vaccination and died shortly afterwards. There was no observable inflammation and/or reactions at the site of injection in fish which died or lived following injection.

Since i.p. injection of 50 µg flagellin/fish did not cause death, nor was it apparently detrimental to fish health, this concentration was considered to be safe for use on larger scale vaccinations (below).

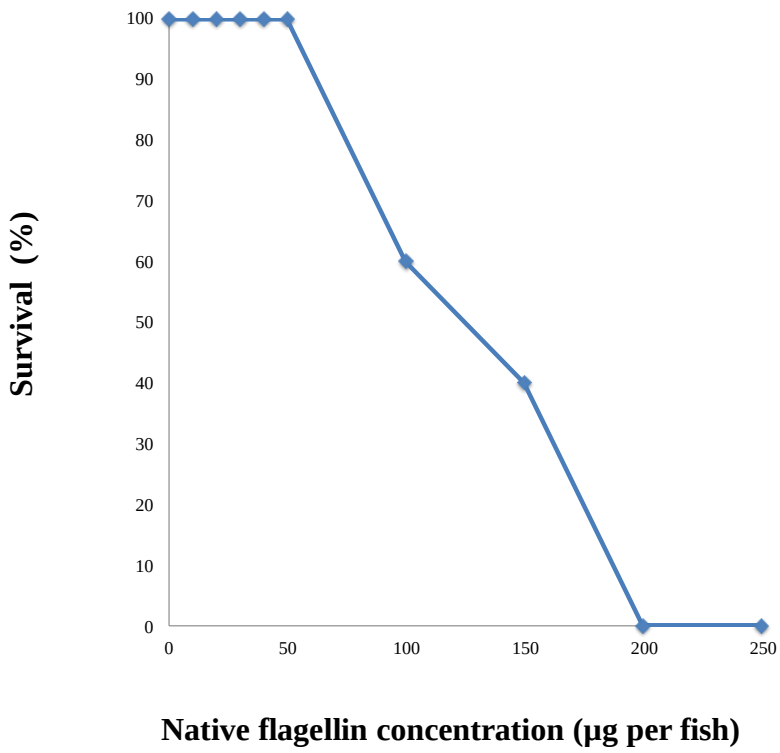


Fig 3.7: Toxicity of native *Y. ruckeri* flagellin to naïve rainbow trout over a range of concentrations.

3.8 Protection conferred by native *Y. ruckeri* flagellin

The protective properties of native *Y. ruckeri* flagellin protein for rainbow trout against challenge with a serotype O1 (YR1) or EX5 (R1) *Y. ruckeri* strain were determined. It should be noted that experiments regarding flagellin as a sub-unit vaccine were carried out alongside vaccination/challenge studies regarding flagellin as part of a whole-cell vaccine (Section 3.5). Rainbow trout (average weight = 5 to 6

g; N = 80) were sedated in anaesthetic and i.p. vaccinated with native flagellin (50µg/fish). As previously stated, controls were mock-vaccinated with sterile PBS (N = 60). All fish recovered from anaesthesia within a matter of minutes and showed no signs of disease or distress. After 28 days post-vaccination, half of the flagellin-vaccinates (N = 40) were i.p. challenged with the serotype O1 (YR1) strain (8×10^5 cells/ml), whereas the other half (N = 40) were i.p. challenged with the EX5 (R1) isolate (4.5×10^5 cells/fish). Fish mock-vaccinated with PBS were either challenged with the serotype O1 (YR1) (8×10^5 cells/ml; N = 25) or EX5 (R1) (4.5×10^5 cells/fish; N = 25) strain. A further control group of trout mock-vaccinated with PBS (N = 10) were mock-challenged with PBS. Prior to i.p. injection, all fish were again sedated in anaesthetic and fully recovered from anaesthesia. Survival rates for all groups 14 days post-challenge are outlined in Fig 3.8.

All fish administered with native flagellin (N = 80) after 14 days post-challenge survived (RPS = 100%) (Fig 3.8). Furthermore, fish injected with flagellin showed excellent signs of health and behaviour compared to control fish surviving challenge. Protection was strain-independent as half (N = 40) were challenged with the serotype O1 (YR1) isolate, whereas the second half (N = 40) were challenged with the EX5 (R1) strain. These results are significant insofar as native flagellin can protect against both a flagellin-producing (i.e. serotype O1) (Fig 3.4; Lane 6) and flagellin-devoid (i.e. EX5) (Fig 3.4; Lane 7) *Y. ruckeri* strain.

Post-mortem examination of fish which succumbed to disease following bacterial challenge all showed signs of haemorrhaging and liquefaction of internal tissues. Bacterial cells were recovered from internal tissues (e.g. kidney, spleen and ascetic fluid) as pure cultures and were confirmed to be *Y. ruckeri* serotype O1 (YR1) or EX5 (R1) depending on the strain used for challenge.

Overall, these results demonstrate that native flagellin can be an efficacious vaccine in preventing ERM in rainbow trout. The ability to protect against a strain which does not produce this protein (i.e. EX5) also suggests that it may be a potent non-specific vaccine. Thus, the next rational step in this study was to investigate the possibility of cloning/overexpressing the protein encoding *Y. ruckeri* flagellin for use as a recombinant vaccine.

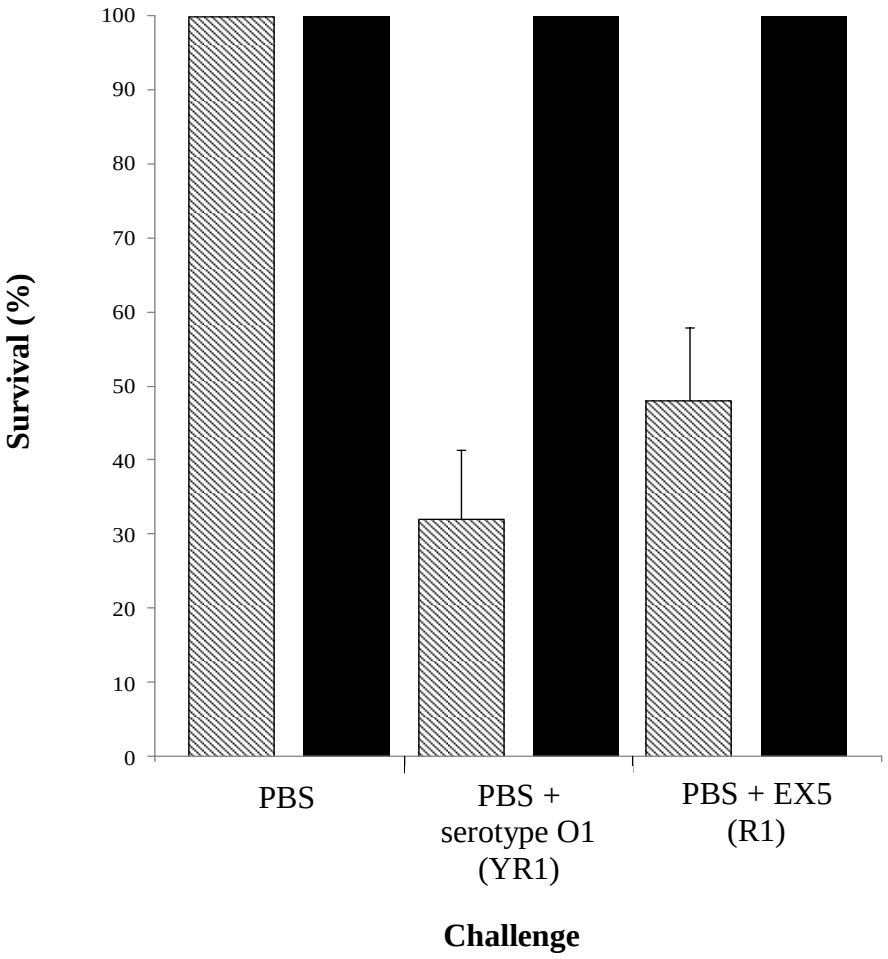


Fig 3.8: Use of native flagellin as a vaccine (50 µg/fish) to protect rainbow trout against challenge with a serotype O1 (YR1) and EX5 (R1) *Y. ruckeri* strain. Fish were mock-vaccinated with PBS (▨) or immunized with PBS + native *Y. ruckeri* flagellin (■) 28 days before challenging. Vaccinates were then mock-challenged with PBS only (control) or challenged with PBS + live serotype O1 (YR1) or EX5 (R1) bacteria. Survival rates were recorded 14 days post-challenge. Standard errors, where applicable, are included.

3.9 Cloning of the *Y. ruckeri fliC* gene

The *Y. ruckeri* genome has been sequenced, although it is currently lacking annotation and is available only as short sequence reads (NCBI Accession: PRJNA55249; ID: 55249). Consequently, the gene encoding the *Y ruckeri* flagellin monomer (termed *fliC*) could not be readily located. Moreover, the *Y. ruckeri fliC*

gene has not been described within the literature. Still, the sequence of a 185 amino acid (aa) peptide from *Y. ruckeri*, which was considered to be a "flagellin-like" protein, was available within the NCBI protein database (GenBank accession No: ZP_04618012.1). Protein BLAST analysis of the peptide sequence showed that it shared a high level of protein identity (90%) to that of *Y. enterocolitica* flagellin. BLAST analysis of the gene encoding *Y. enterocolitica* flagellin (*fliC*) (GenBank accession No: ADB96220.1) found it to be similar to other *Yersinia* spp., particularly at both the N- and C- terminals. Similar sequences were also found within the genomic library of the *Y. ruckeri* strain ATCC 29473, suggesting that the gene of interest was not dissimilar to that of other *Yersinia*. Therefore, primers containing restriction sites for *Xho*I and *Nde*I could be designed based upon conserved N- and C- terminal sequences to amplify the *fliC* gene of *Y. ruckeri*.

A PCR reaction using Pfu enzyme and genomic DNA from the serotype O1 (BA19) strain as a template, with the primers FLA-F/FLA-R, resulted in a single product ~1200 bp in size (Fig 3.9). This PCR product (termed *fliC*) was purified, digested with the restriction enzymes *Xho*I and *Nde*I, and cloned into vector pET28-b to create the plasmid YRF1 (pET28-b + *fliC*). The ligation mixture was then used to transform chemically-competent *E. coli* XL1-Blue bacteria. Six colonies were picked and grown overnight before purifying plasmid DNA from each culture. Purified plasmids were digested with the restriction enzymes *Nde*I and *Xho*I (Fig 3.10). Of the six plasmids digested, two (Fig 3.10; Lanes 3 and 4) gave a detectable band of ~1000 bp, indicating that the *fliC* PCR product was correctly inserted within the pET28-b vector. The *E. coli* XL1-Blue transformant, containing the YRF1 plasmid shown in Fig 3.10 (Lane 3), was termed *E. coli* XL1-Blue/YRF1. This strain was stored as glycerol stocks at -70ºC and used in all subsequent experiments as a source of YRF1.

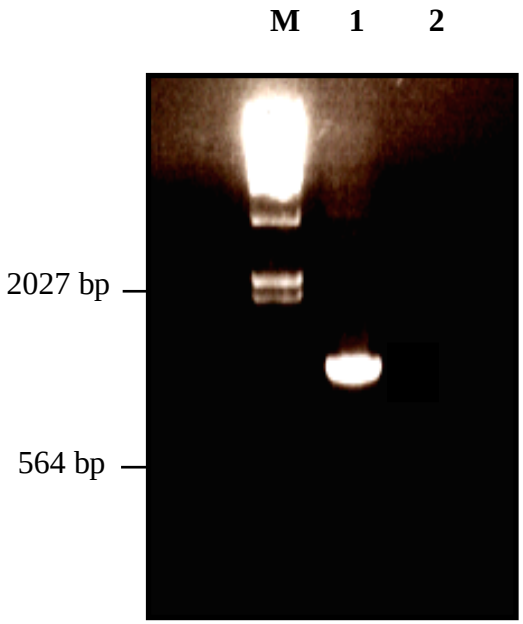


Fig 3.9: PCR amplification of the serotype O1 (BA19) *Y. ruckeri* flagellin (*fliC*) gene using primers (FLA-F and FLA-R). Primers were based on conserved N- and C-terminal sequences of the *Y. enterocolitica* flagellin gene. **M:** Molecular weight marker (λ *Hin*dIII; Fermentas). **Lane 1:** PCR product using *Y. ruckeri* genomic DNA as a template. **Lane 2:** Negative PCR control. A detectable PCR product of ~1200 bp, the estimated size of *fliC*, is visible in lane 1.

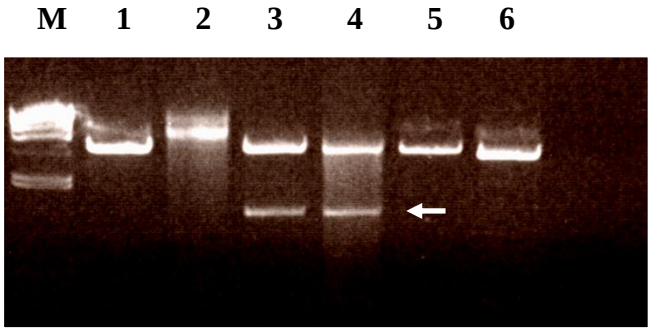


Fig 3.10: Screening of six *E. coli* XL1-Blue transformants for pET28-b plasmid with a *Y. ruckeri* flagellin gene (*fliC*) insert (YRF1). Plasmids were purified via the alkaline lysis method before digesting overnight with *Xho*I and *Nde*I at 37°C. **M:** Molecular weight marker (λ *Hin*dIII). **Lanes 1-6:** Digested plasmids isolated from XL1-Blue *E. coli* transformants. DNA fragments in lanes 3 and 4 gave a detectable fragment of approx.1000 bp (indicated), indicating that the *Y. ruckeri fliC* gene was incorporated into the plasmid in the correct orientation.

3.10 Sequencing the YRF1 plasmid insert

The YRF1 plasmid from the *E. coli* XL1-Blue/YRF1 transformant was sequenced using the FLA-F and FLA-R primers. Additional sequences adjacent to original primers were obtained by sequencing with the internal primers FLG-L and FLG-R. Each sequencing reaction was repeated to ensure that the DNA sequence was correct. The final DNA sequence, along with the predicted amino acids encoded by the open reading frame (ORF), is available in the appendix (Fig 5.7). The ORF also includes 5' and 3' sequences from the pET-28b vector.

Analysis of the *fliC* DNA sequence showed that the gene has an overall G+C content of 52.6%, a number which is not dissimilar to that predicted for the *Y. ruckeri* genome (Ewing *et al.*, 1978). The deduced protein sequence (Fig 5.7; B) consists of 452 amino acids and has an estimated molecular weight of 46.77 kDa. The protein also has histidine (His) tags successfully incorporated at both the N- and C-terminal. BLAST analysis of this sequence showed that it is similar (69% identity) to that of the *Y. enterocolitica* 39.6 kDa flagellin protein (Fig 3.11). A tree of the flagellin monomer amino acid sequence (Fig 3.12) could be constructed using the online phylogeny program (http://www.phylogeny.fr/). What is apparent from this tree is that the amino acid sequence of *Y. ruckeri* flagellin is most similar to flagellin of other *Yersinia* spp. Some degree of amino-acid relatedness was also shown between *Y. ruckeri* flagellin and that of *Serratia*, *Salmonella* and *E. coli* flagellins (Fig 3.12). However, the 39.6 kDa flagellin monomer from *Y. enterocolitica* is the most closely related flagellin species. In particular, amino-acid sequences were highly conserved at the N- and C-terminals between both proteins (Fig 3.11). Using the web based Sequence Annotated by Structure program (SAS) (http://www.ebi.ac.uk/thornton-srv/databases/sas), the secondary structure of both *Y. ruckeri* and *Y. enterocolitica* flagellin proteins were predicted to be helical at each terminal, with some variability in the central sequences (Fig 3.13). At this stage therefore it was established that the gene (*fliC*) encoding the *Y. ruckeri* flagellin monomer (FliC), or at least a gene encoding a protein with significant identity to flagellin, was successfully cloned within the pET28-b vector. Therefore, the YRF1 construct could be used to overexpress recombinant *Y. ruckeri* flagellin (r-flagellin).

3.11 Presence of the flagellin (*fliC*) gene within the *Y. ruckeri* EX5 genome

Interestingly, a *fliC* PCR reaction which was performed using genomic DNA from the *Y. ruckeri* EX5 (R1) isolate also generated a PCR product (Fig 3.14) of the same size as that produced when using serotype O1 (BA19) DNA. This shows that the EX5 group, or at least an EX5 strain described by Austin *et al.* (2003), still carries the *fliC* gene within the genome despite it being unable to express this protein (Fig 3.4; Lane 2 and 6).

```
21    MAVINTNSLSLLTRHLNKSQGSLGTAIERLSSGLRINSAKDDAAGQAIANRFTSNINGLT80
1     MAVINTNSLSLLTQNLNKSQSSLGTAIERLSSGLRINSAKDDAAGQAIANRFTSNING-T59

81    QAARNANDGISLSQTAEGALGEINNNLQRVRDLTVQAQNSSNSASDIDSIQSEVNQRMEE140
60    VAARNANDGISLSQTAEGALGEINNNLQRVRDLTVQAQNSSNSASDIDSIQSEVNQRMEE119

141   INRVTKQTDFNGIKVLDNRTAANAEYAFQVGSQDAQKINIEIGSSAGWNLATAGAGGTSS200
120   INRVTKQTDFNGIKVLDNRTATDSSYDFQVGSKDNEQISIAIGASSGWNLAAAGAGGISG179

201   DVVNDSTQISKAKETVVQTLSGKTEAQINTALTKFTTDVKAATDAAGVV-TAKGALTTAL259
180   DTIN------TYKFTTT-----------TALKTAQDNVKTATDDVPVKQKAYEDAVAAD221

260   GLKADADLGTAVSSAAFGTDLSVDQIAGVKSGVYSAAINGANYATAKTEAEVSAAQAGAK319
222   PADAERALKTAWDTAK--------TLVTTNTGLYNTALK---TATATGEA----------260

320   TAGAMVNGNFRSVEAKGFDVLKGNVTGGATGTATGTTPLADIDAALKAVDSQRSSLGASQ379
261   -----VNGNARTVAAEGFDVLKGQV--AADGTAAGTTPLADIDKALKAVDTQRTGLGASQ313

380   NRFESTITNLNNTVNNLTSARSRIQDADYSTEVSNMSRAQILQQAGTSVMAQANQVPQTV439
314   NRFESTITNLNTTGNNLTSARSRIQDADYSIEVSNMSRAQILQQAGISVLAQANQVPQIV373

440   LSLLR  444
374   LSLLR  378
```

Fig 3.11: Alignment of the predicted amino-acid sequence of *Y. ruckeri* flagellin (FliC) with that of the 39.6 kDa flagellin protein from *Y. enterocolitica* (PIR: S69767). The amino acid sequence of *Y. ruckeri* flagellin is shown in bold. Conserved areas shared by the two proteins are highlighted in grey.

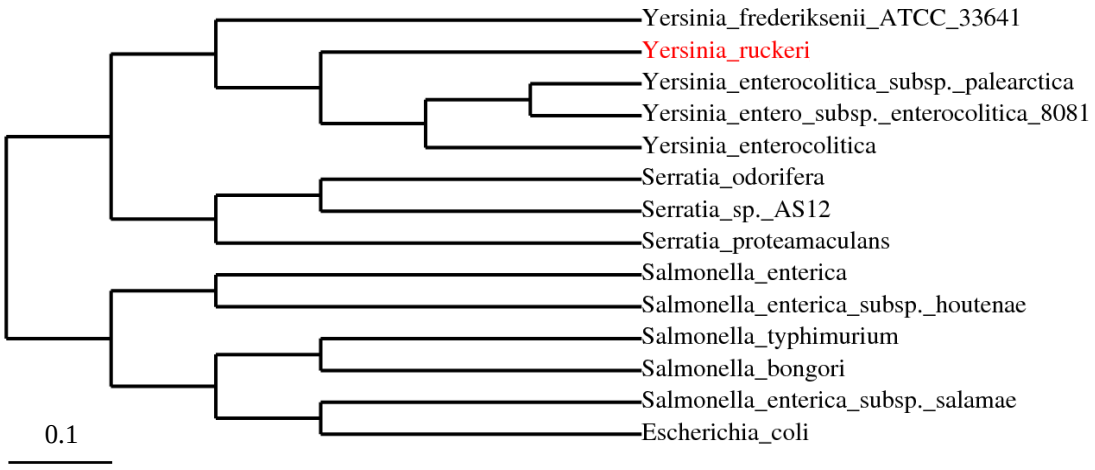
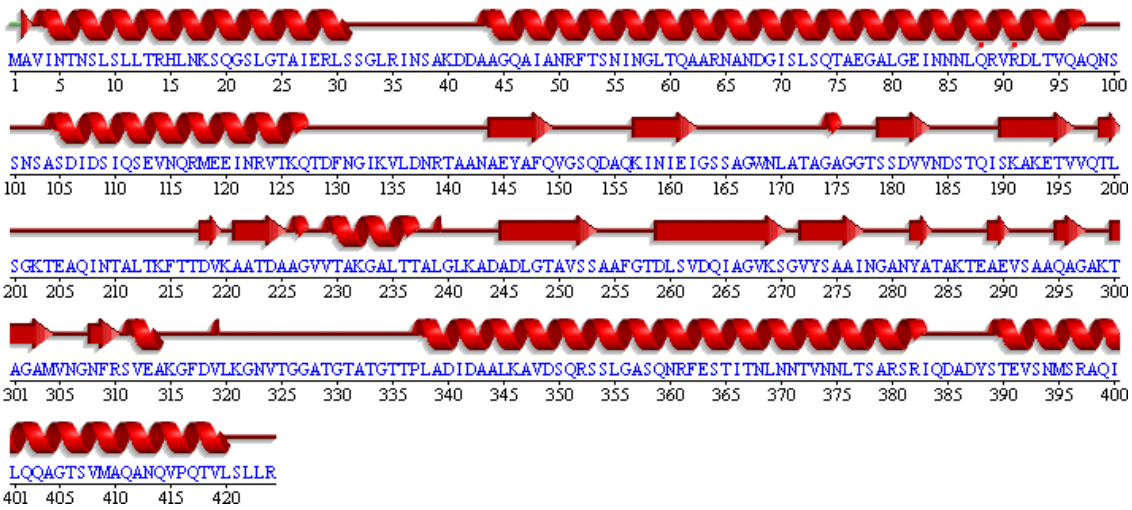


Fig 3.12: Tree showing the relatedness of peptide sequences of proteins similar to the *Y. ruckeri* flagellin (FliC) protein based on amino acid sequence similarity.

Y. ruckeri flagellin



Y. enterocolitica flagellin

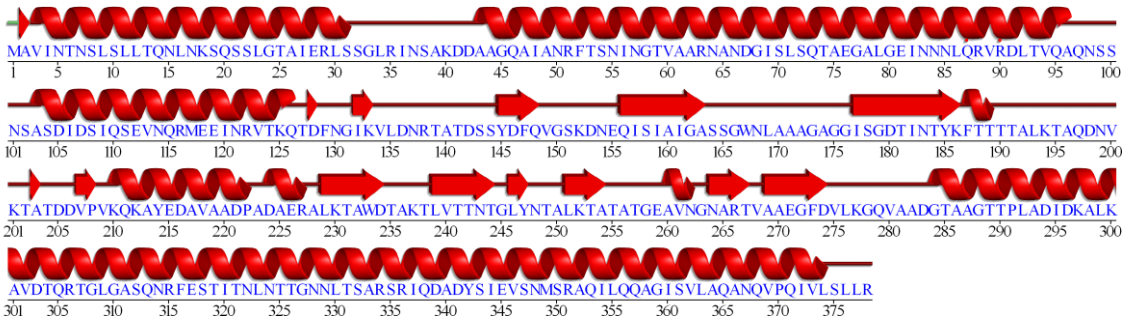


Fig 3.13: Predicted secondary structure of *Y. ruckeri* and *Y. enterocolitica* flagellin proteins. Note that both proteins are helical at the N- and C- terminals.

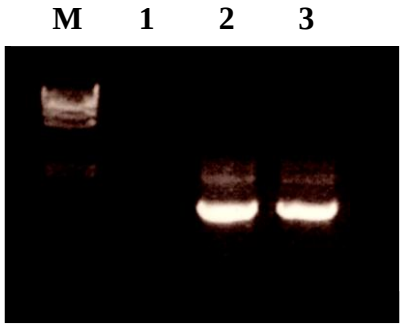


Fig 3.14: PCR amplification of the flagellin (*fliC*) gene using genomic DNA from a *Y. ruckeri* EX5 (R1) isolate. **M:** Molecular weight marker (λ *Hin*dIII). **Lane 1:** Negative control (dH_2O only). **Lane 2:** Positive control using genomic DNA from a serotype O1 (YR1) *Y. ruckeri* strain. **Lane 3:** PCR reaction using EX5 (R1) genomic DNA as template. A PCR product, in addition to the positive control (lane 2), was generated for the reaction using genomic DNA from the EX5 (R1) strain (lane 3).

3.12 Overexpression and purification of recombinant *Y. ruckeri* flagellin (r-flagellin)

All results regarding the induction and purification of recombinant flagellin (r-flagellin) are shown in Fig 3.15. After confirming that the *Y. ruckeri* flagellin gene was cloned (above), the YRF1 construct was used to transform the *E. coli* BL21 DE3 expression strain. Prior to induction with IPTG, the *E. coli* BL21 DE3 transformant (*E. coli* BL21 DE3/YRF1) is either expressing low levels of r-flagellin or perhaps endogenous *E. coli* flagellin (Fig 3.15; B: Lane 2). However, the addition of 1 mM IPTG significantly induced expression of r-flagellin (Fig 3.15; B: Lane 3), predominately as insoluble inclusion bodies (IBs) (Fig 3.15; B: Lane 5). A moderate amount of soluble r-flagellin was also present (Fig 3.15; B: Lane 4), although preliminary attempts to purify the His-tagged protein by binding to an IMAC column were initially unsuccessful. Nonetheless, the recombinant protein could be cleaned and purified by exploiting the insoluble nature of inclusion bodies.

It is estimated that the production of r-flagellin, under the current conditions of induction (see Materials and Methods), was in the range of 40 mg of protein/L. This is approximately 8 times more than what could be obtained from 6 L of bacterial culture using the acid disassociation and re-association (Section 3.6). By varying culture and induction conditions it was later shown that growing the *E. coli* BL21 DE3/YRF1 transformant at 28ºC ($OD_{600} \geq 1.5$), before inducing with 0.1 mM IPTG for 4 h, can significantly increase the amount of soluble flagellin without adversely affecting overall flagellin yield. Preliminary tests have also shown that this soluble r-flagellin could be purified using an IMAC column (Fig 3.16; B).

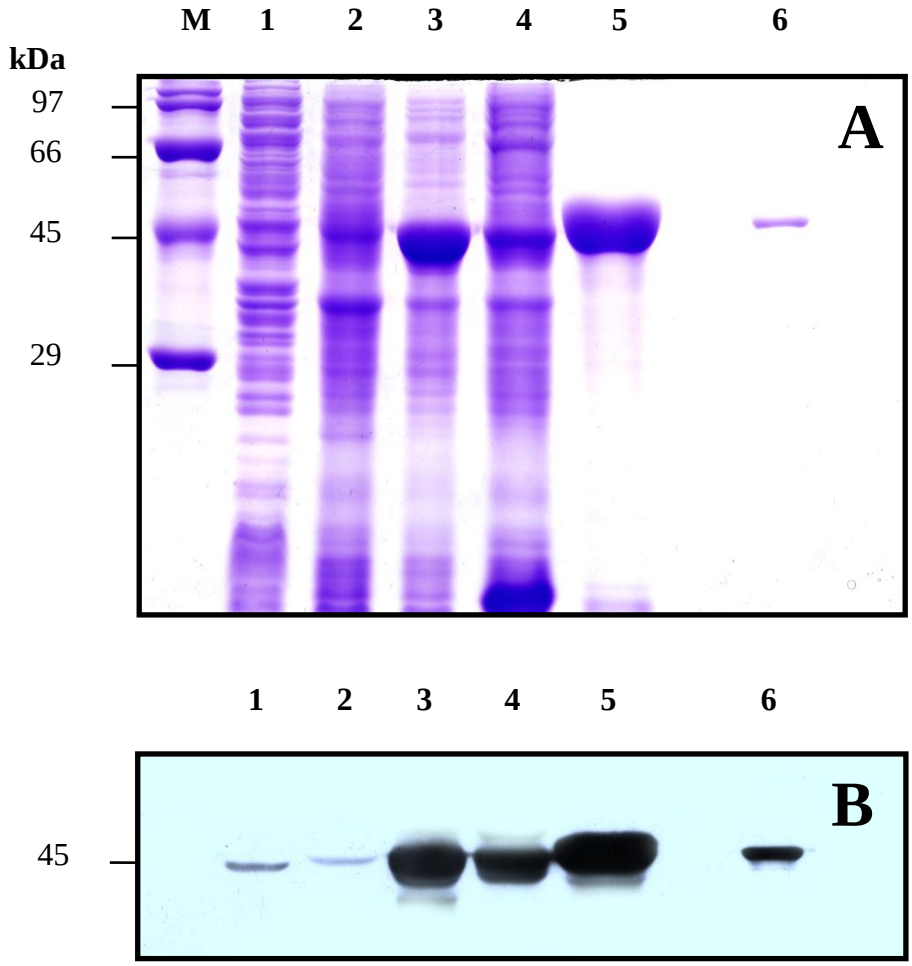


Fig 3.15: Induction, overexpression and purification of recombinant *Y. ruckeri* flagellin (r-flagellin). **A:** Proteins fractionated on a 10% (w/v) SDS-PAGE gel. **B:** Immunodetection of flagellin with the monoclonal antibody #15D8. **Lane 1:** Whole-cell proteins (WCPs) of a motile serotype O1 *Y. ruckeri* strain (BA19) used to source the flagellin (*fliC*) gene. **Lane 2:** WCPs of the *E. coli* BL21/YRF1 transformant before induction. **Lane 3:** WCPs of BL21/YRF1 4 h after induction with 1 mM IPTG. **Lane 4:** Soluble fraction of induced cells. **Lane 5:** Insoluble fraction of induced cells; **Lane 6:** Final r-flagellin product (1 µg) after further purification steps (see Materials & Methods) used to vaccinate fish.

To ensure that the final r-flagellin product (Fig 3.15) was relatively pure and without major contaminants (e.g. other proteins or LPS), the sample was again separated on a 10% SDS-PAGE gel and visualised by staining with Coomassie brilliant blue R250 or silver nitrate (Fig 3.16; A). Aside from the recombinant protein, no other protein or carbohydrate bands were observed within the gels. Although this does not rule out the potential presence of very low level contaminants which may not be visible on the SDS-PAGE gels, it does suggest that it is of sufficient purity for use in fish trials.

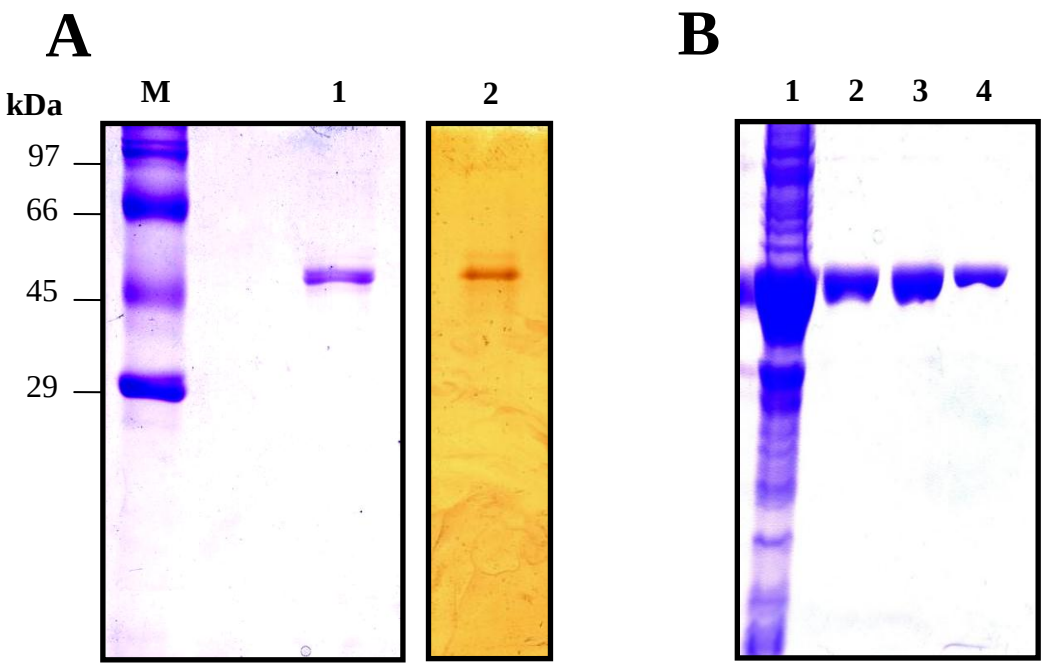


Fig 3.16: SDS-PAGE analysis of r-flagellin preparations. After running samples on a 10% (w/v) SDS-PAGE gel, protein were stained with Coomassie brilliant blue-R250 (**A; Lane 1** and **B**) or silver nitrate (**A; Lane 2**) following electrophoresis. R-flagellin was purified from IBs (**A**) or by passing r-flagellin (expressed as soluble protein) through an IMAC column (**B**). **Lane A; 1:** r-flagellin (5 µg). **A; Lane 2:** r-flagellin (20 ng). **B; Lane 1:** Soluble fraction of induced *E. coli* BL21-DE3/YRF1. **B; Lane 2-4:** IMAC elutes 1 (5 µg), 2 (5 µg) and 3 (2 µg). **M:** Molecular weight marker. See text for details.

3.13 Toxicity of r-flagellin to rainbow trout

Since native *Y. ruckeri* flagellin (Section 3.7) caused fish mortality at higher levels, it was considered vital that r-flagellin was also administered to fish (average weight = 5 to 6 g) over a range of amounts (0, 10, 25, 50, 100, 150, 200 µg/fish) before commencing large-scale vaccinations in order to test for toxicity. For each test group, at least 5 fish were injected with each amount. After anaesthesia, each preparation was administered in volumes of 100 µl. All fish had recovered from anaesthetic within 15 min and showed no signs of distress. After 48 h, all fish remained alive (100% survival), again showing excellent signs of health. Moreover, there was no sign of inflammation or discolouration on any fish at the point of injection, nor were there any observable changes between the internal organs of test or control fish (i.e. those which did not receive injections). In this respect, it is likely that *Y. ruckeri* flagellin was not the cause of death in fish previously administered with the native preparation since all fish administered with the recombinant protein survived.

One explanation as to why the native preparation killed fish at high doses is the presence of additional molecules and/or compounds which are toxic for fish at higher concentrations (e.g. LPS). Silver staining of both native and recombinant protein was performed to detect any contaminating material (Fig 3.17). Whereas the r-flagellin preparation was again shown to be pure, a low molecular weight band was present within the native preparation. This may be low molecular weight proteins or the lipid A region of LPS. However, the characteristic "smear" of carbohydrate from LPS was not detectable, suggesting that LPS is not a major contaminant. Contamination with other proteins was considered to be minimal.

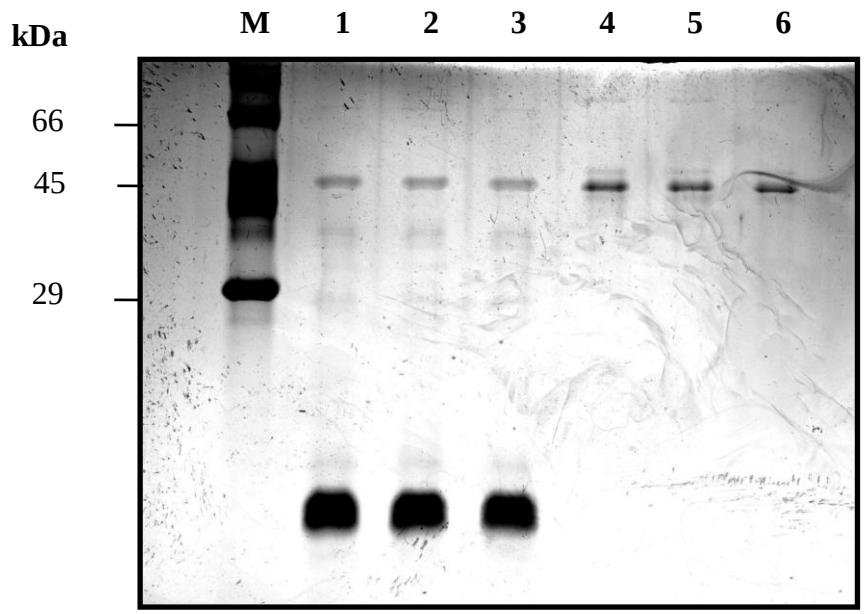


Fig 3.17: Silver staining of native and recombinant *Y. ruckeri* flagellin (r-flagellin). Proteins were fractionated on a 12% (w/v) SDS-PAGE gel before staining proteins with silver nitrate. **M:** Molecular weight marker**. Lanes 1 to 3:** Native flagellin (20 ng). **Lanes 4 to 6:** r-flagellin (20 ng).

A notable difference between fish administered with r-flagellin or native flagellin was the rate by which fish recovered from anaesthetic. Whereas trout administered with r-flagellin recovered within a matter of minutes, fish injected with higher concentrations of native flagellin failed to fully recover before dying. Thus, r-flagellin could be safely administered to fish at relatively high concentrations without any detrimental effects to health.

3.14 Protection conferred by r-flagellin

The protective effect of r-flagellin against *Y. ruckeri* infections for rainbow trout (average weight = 5 to 6 g) was investigated. Groups of 25 fish were i.p. injected with 0, 10, 25 and 50 µg of r-flagellin in PBS (100 µl/fish). Fish fully recovered from anaesthesia and showed excellent signs of health. After 28 days post-vaccination, trout were again anaesthetised before challenging with the serotype O1 (YR1) (8×10^5 cells) or EX5 (R1) (4.5×10^5 cells/ml) strains. All fish recovered from anaesthesia.

Results from this challenge are shown in Fig 3.18. After 48 hours, 92 and 94% of all control fish had died after challenging with the serotype O1 (YR1) and EX5 (R1) strain, a number which was much higher than that was expected to occur over a period of 2 weeks (50-70% mortality). The challenge dose (supposedly 8 x 10^5 cells and 4.5 x 10^5 cells) was probably higher than expected, thus an explanation is that the bacterial counts were incorrect. Nonetheless, the levels of survival were proportionate to the amount of antigen administered insofar as trout injected with 10, 25 or 50 μg of r-flagellin showed a survival rate of 38, 63 and 73% after challenging with the EX5 (R1) strain. This equates to RPS values of 33, 60 and 72%, respectively. Similarly, r-flagellin vaccinated trout challenged with the YR1 isolate displayed survival rates of 60, 52 and 68%. This corresponds to RPS values of 59, 50 and 67%, respectively.

It is evident from these results that protection conferred by this protein is dose-dependent. However, given the severity of challenge against control groups, the experiment was terminated after 48 h.

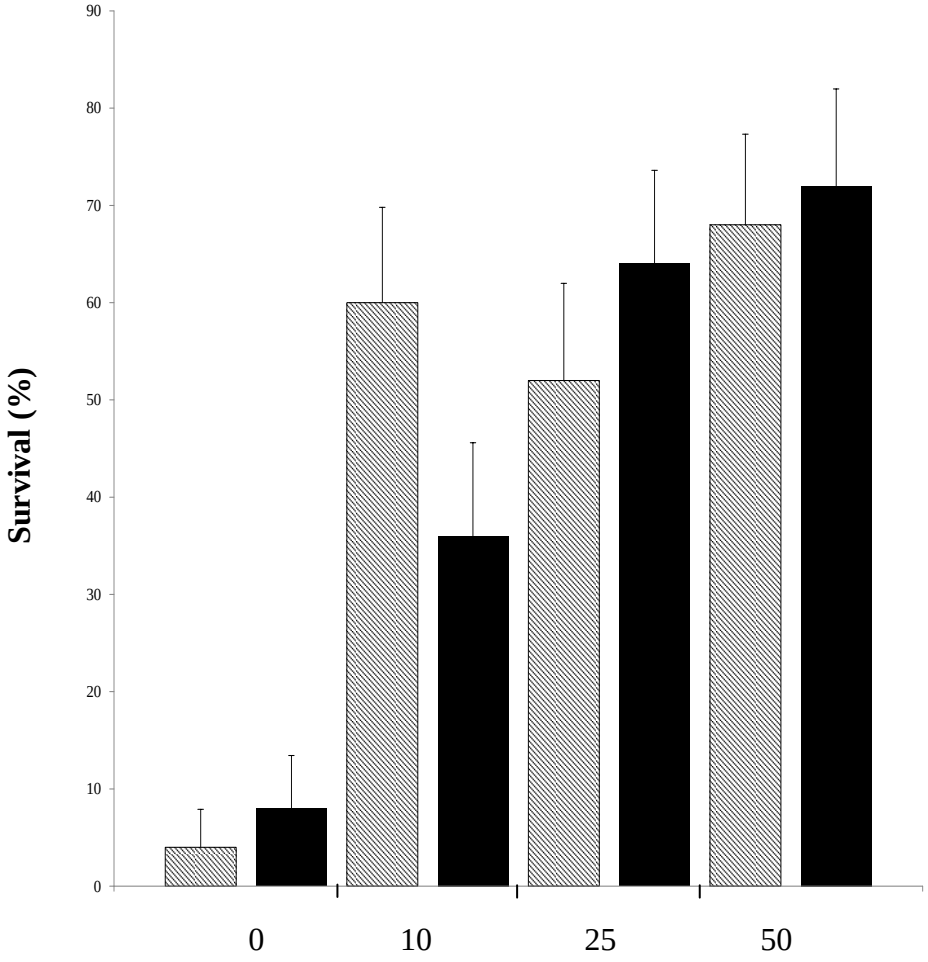


Fig 3.18: Survival rates of rainbow trout 48 h after challenge with a *Y. ruckeri* serotype O1 (YR1) (▨) or EX5 (R1) (■) isolate. Bacterial challenge was performed 28 days post-vaccination. Standard error values are included (N = 25).

3.15 Onset of protection and estimation of the minimal protective dose with r-flagellin

As the challenge dose was considered to be too high, the experiment was repeated. Furthermore, since protection could be obtained after 28 days post-vaccination, challenge was performed after 14 days to determine if protection was conferred after a shortened period of time. In this case, only the *Y. ruckeri* EX5 (R1) strain was used for challenging fish.

Groups of non-vaccinated rainbow trout (average weight = 5 to 6 g) were administered with a low range of r-flagellin (0, 10, 25 and 50 µg/ fish in 200 µl PBS). The highest concentration of r-flagellin tested was again 50 µg/fish as this concentration sufficiently protected against bacterial challenge when using the native protein as a vaccine (Section 3.6). Each group consisted of 20 fish (N = 20) and were anaesthetised prior to administering the vaccine by i.p. injection. All fish fully recovered from anaesthesia and resumed feeding. No observable discoloration and/or inflammation occurred at the site of injection. Livestock were maintained in the aquarium, without disturbing, for 14 days as opposed to 28 days. Fish were then sedated in anaesthetic before injecting freshly grown *Y. ruckeri* EX5 [R1] cells (1 x 10^5 bacterial cells/fish). All fish fully recovered from anaesthesia and were closely observed for 7 days post-challenge.

Results from this challenge are shown in Fig 3.19. In this case, all fish administered with r-flagellin survived (RPS = 100%), whereas those mock-vaccinated with PBS showed mortalities of 30%. Many of those which died showed characteristic signs of ERM (Fig 3.20) including haemorrhaging around/within the oral cavity and exophthalmia. Other symptoms included the darkening of skin and internal scaring/liquefaction of tissues. Although not shown in Fig 3.20 other signs of disease included the formation of petechial haemorrhaging around the fins, gills and anus. Infected fish also produced excessive amounts of mucus. When taking any of these symptoms into consideration, in combination with the number of fish which died, 75% of all fish showed disease-related symptoms (Fig 3.21). On the other hand the majority of r-flagellin vaccinated fish (59/60) showed excellent signs of health.

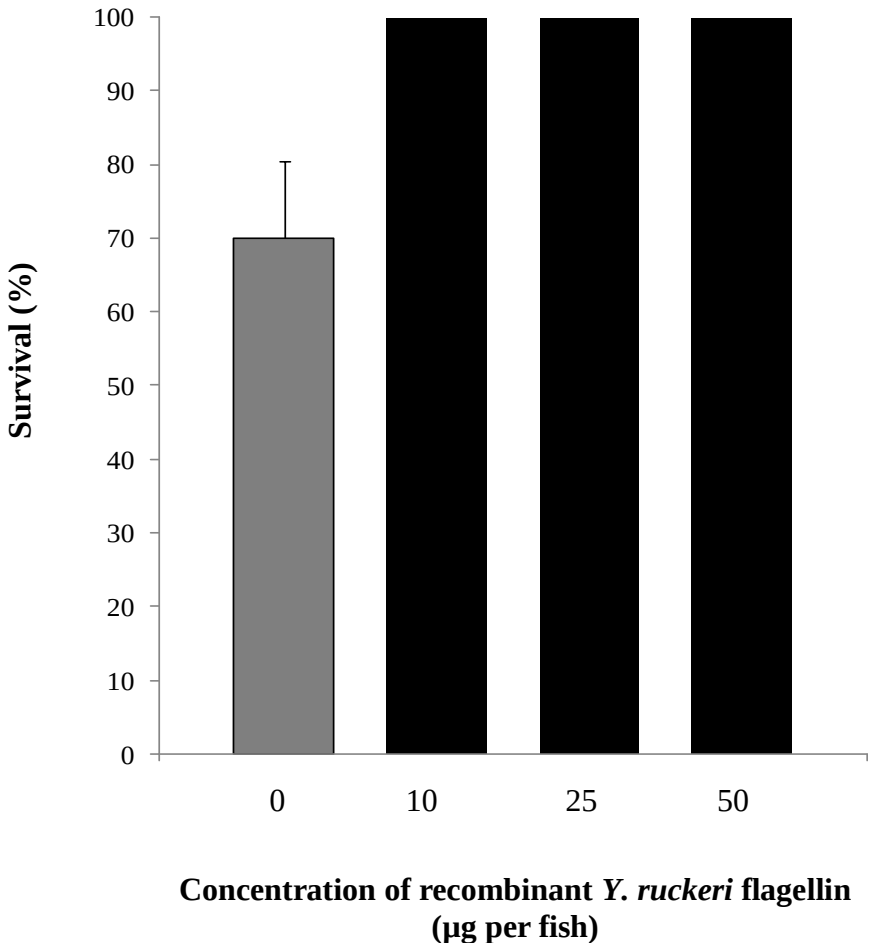


Fig 3.19: Use of *Y. ruckeri* r-flagellin to protect fish against intraperitoneal (i.p.) challenge with the EX5 (R1) isolate. Control fish were i.p. injected with 200µl PBS (■), whereas other groups were vaccinated with various concentrations (10, 25 and 50 µg per fish) of r-flagellin in 200µl PBS (■). After 14-days, fish were i.p. challenged with 4.5 x 10^5 cells of the EX5 (R1) strain. Survival after 7 days post-challenge is shown above. The standard errors of controls are included (N = 20).

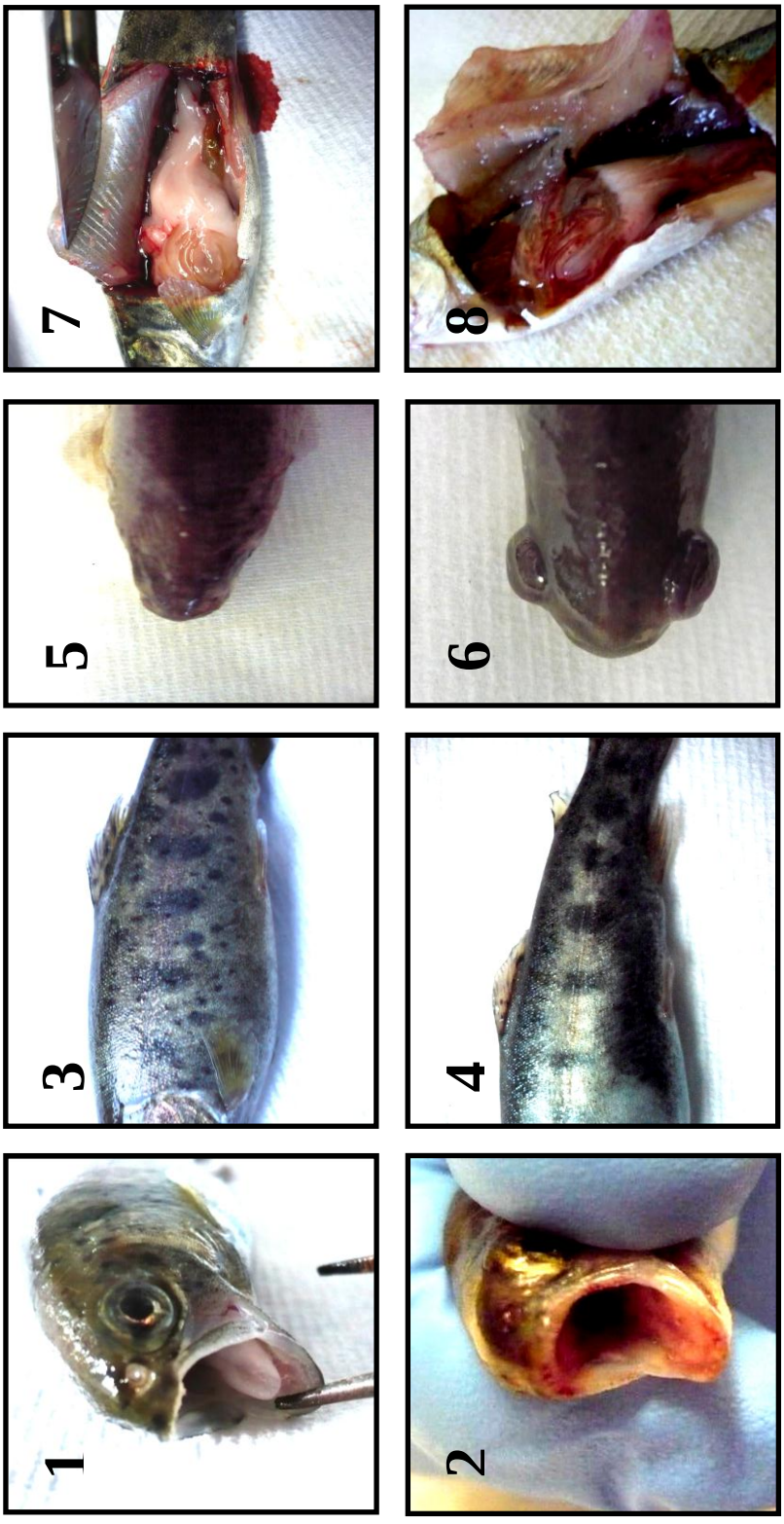


Fig. 3.20: Fish showing some symptoms linked with ERM following i.p. challenge with the *Y. ruckeri* EX5 (R1) strain. Pictures (**2**, **4**, **6** and **8**) show a trout exhibiting some signs of ERM, whereas a healthy (i.e. non-challenged) specimen (**1**, **3**, **5** and **7**) is included for comparisons. Infected trout showed signs of haemorrhaging around the oral cavity (**2**); darkening of the skin (= granular melanosis) (**4**); protrusion of eyes from the ocular socket (= exophthalmia) (**6**) and internal liquefaction (i.e. yellowing) of internal organs (**8**).

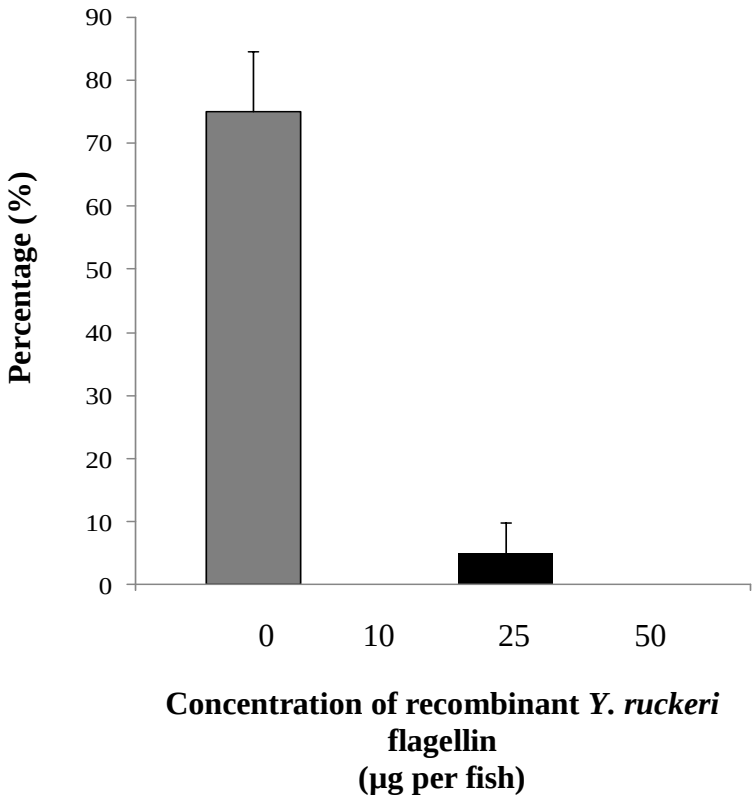


Fig 3.21: Using *Y. ruckeri* r-flagellin to prevent mortalities and/or the development of symptoms associated with ERM after challenging with the EX5 (R1) isolate. Controls were administered with PBS (■) or vaccinated with various concentrations (10, 25 and 50 µg per fish) of r-flagellin (■). The total number of mortalities, in combination with the number of fish showing signs of ERM, is presented above as an absolute percentage. Standard error is shown where appropriate (N = 20).

Interestingly, all r-flagellin vaccinated fish showed heightened levels of hunger compared to the controls. To investigate what effect this may have on fish physiology, all fish were weighed (Fig 3.22; A) and measured (Fig 3.22; B) 7 days post-challenge. Although the mean values of weights and lengths of fish between control and vaccinated groups were not significantly different ($P < 0.05$), it nonetheless shows that the vaccine does not have a detrimental effect to health.

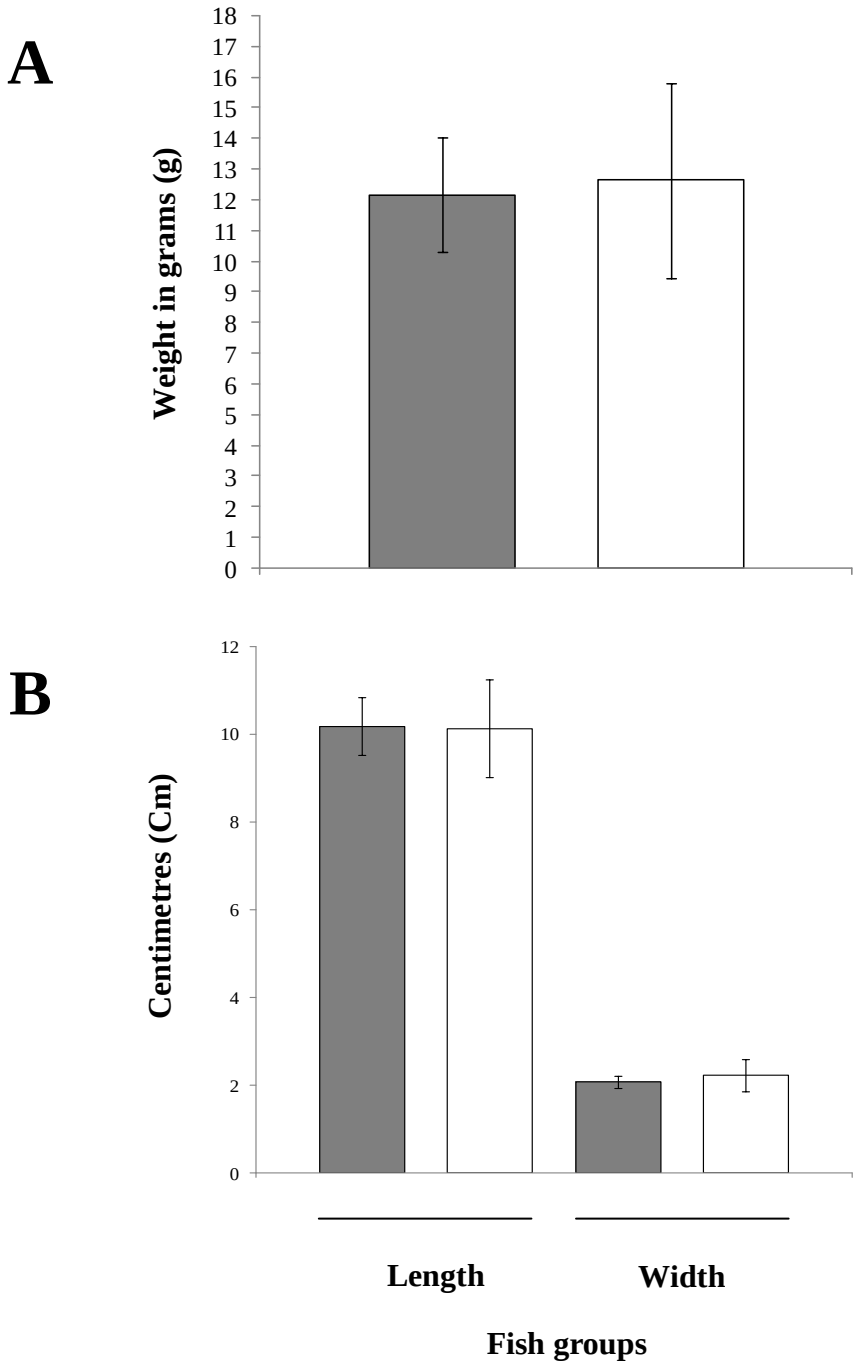


Fig 3.22: Average weight (**A**) and length/width (**B**) of fish surviving 7 days post-challenge with the EX5 (R1) isolate. Data for fish mock-vaccinated (N = 14) with PBS is shown (■), as is that of fish vaccinated with recombinant *Y. ruckeri* flagellin (□) (N = 60). Standard deviation values are included.

Overall, these data demonstrates that the r-flagellin protein, even at low concentrations (e.g. 10 µg/fish), can be an efficacious vaccine for preventing the development of ERM associated symptoms and mortalities. Protection can also be conferred after a relatively short period of time (e.g. 14 days). In addition, the mode of protection appears to be non-specific insofar as the *Y. ruckeri* EX5 (R1) strain used to challenge vaccinates is not producing the flagellin molecule used as a vaccine (Fig 3.4; Lane 6).

3.16 Investigating the EX5 (R1) strain for motility and flagellin production

Although the *Y. ruckeri* EX5 (R1) strain does not produce detectable flagellin under standard culturing conditions (Fig 3.4; Lanes 2 and 6), this does not rule out its production under conditions which are known to induce virulence, especially since the *fliC* gene is present within the genome (Fig 3.14). Furthermore, these conditions are known to be somewhat similar to the conditions encountered by the pathogen inside the host. Thus experiments were carried out to investigate if motility and flagellin expression could be induced in EX5 by varying culture conditions.

The *Y. ruckeri* EX5 (R1) strains grown at 28˚C in TSB were non-motile. Culturing over a range of temperatures (e.g. 17, 20, 25, 30˚C) in TSB, with or without the iron chelator 2, 2'-dipyridyl (100 µM), did not influence the non-motile phenotype. This shows that motility is not induced under conditions which are known to influence virulence-gene expression. In addition, EX5 (R1) isolates remained non-motile irrespective of culturing conditions even when recovered from dead or moribund fish.

Western blots using WCPs from the EX5 (R1) isolate, which was again grown under iron-limiting and/or temperature limiting conditions (17ºC as opposed to 28ºC), are shown in Fig 3.23. Hybridizing with the anti-flagellin antibody (#15D8) similarly demonstrated that flagellin was not produced by the EX5 (R1) strain under any of the conditions known to induce virulence. Taking these results into consideration, it is unlikely that flagellin would be expressed within the host.

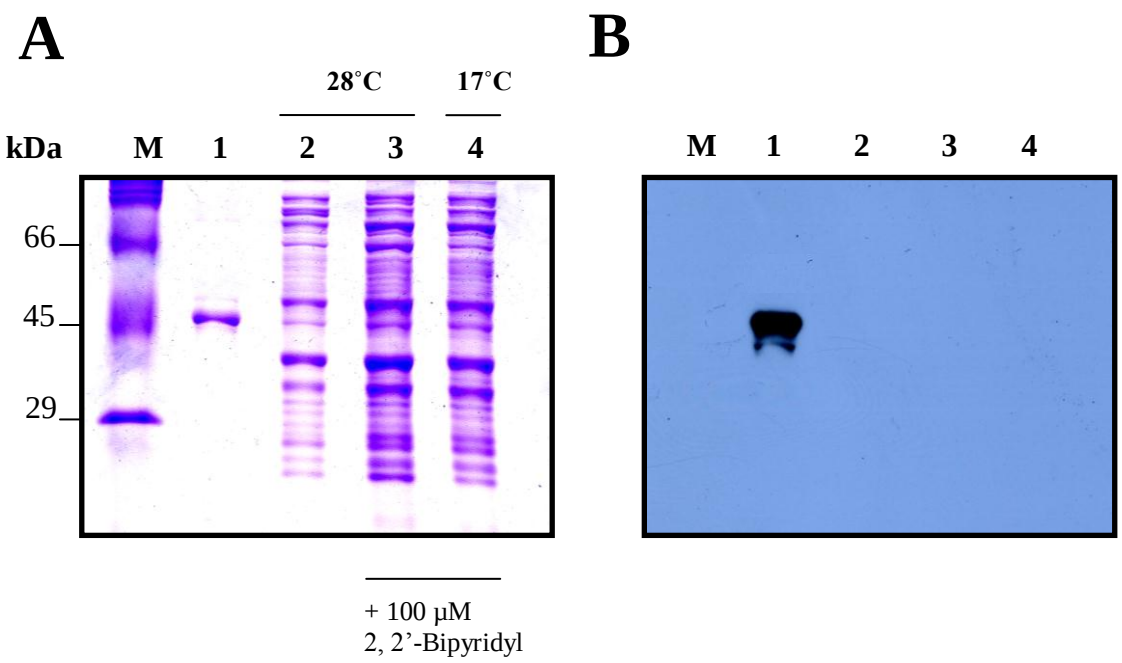


Fig 3.23: Confirmation that the *Y. ruckeri* EX5 (R1) strain does not produce detectable flagellin under iron-limiting and/or temperature limiting conditions. Crude WCP of the *Y. ruckeri* EX5 (R1) strain was separated on a 10% SDS-PAGE (**A**) after growing overnight in TSB at 28˚C (**Lanes 2 and 3**) or 17˚C (**Lane 4**). Cells were grown in the absence (**Lane 2**) or presence (**Lanes 3 and 4**) of the iron chelator 2, 2'-bipyridyl (100 µM). Proteins were blotted onto a nitrocellulose membrane before hybridizing with the anti-flagellin antibody (**B**). Only the recombinant flagellin is detectable (= positive control), whereas no flagellin is detected in any of the EX5 (R1) WCP preparations. **Lane 1:** Recombinant flagellin (2 µg) acting as a positive control. **M:** Molecular weight marker.

3. 17 WCP protein profiles of serotype O1 and EX5 *Y. ruckeri* isolates

From work associated with the characterisation of flagellin, it was evidently clear that both EX5 strains (EX5 and R1) used in this study, compared to the serotype O1 *Y. ruckeri* isolates (BA19 and YR1), are overexpressing a protein with an apparent molecular weight between 30 to 40 kDa (Fig 3.4). Indeed, comparing crude WCPs of all available EX5 isolates with that of other serotypes (i.e. serotype O2, O3 and O4) showed that the overexpressed protein of 30 to 40 kDa was only observed in the EX5 isolates. Whereas a limited number of isolates have been included in Fig 3.24 for simplicity, it should be noted that all EX5 strains described by Austin *et al.* (2003) exhibited an overexpressed protein in the range of 30 to 40 kDa as compared to 6 additional serotype O1 strains.. Furthermore, growing strains under virulence inducing conditions (i.e. iron and temperature limiting) had no effect on the expression of this protein by EX5. 2D SDS-PAGE analysis of WCPs from EX5 (R1) and serotype O1 (YR1) cultures grown under iron and temperature limiting condition (Fig 3.25) demonstrated that four major protein spots (30 to 40 kDa) were more abundant in EX5 relative to O1 (Fig 3.25). It should be noted that although only a single gel from each sample is shown in Fig 3.25, the experiment was repeated four times with new samples to ensure that these proteins were indeed overexpressed by EX5 (R1).

Maldi-ToF mass spectroscopy performed on tryptic digests of all four of these spots showed that they were similar to the outer membrane protein A (OmpA) of *Y. enterocolitica*. Specifically purifying outer membrane proteins (OMPs) from serotype O1 and EX5 isolates via the sodium sarkosyl method demonstrated that the overexpressed protein was in fact an outer membrane protein (Fig 3.26). Thus, it is clear from these results that the EX5 *Y. ruckeri* strain is overexpressing the outer membrane porin protein OmpA.

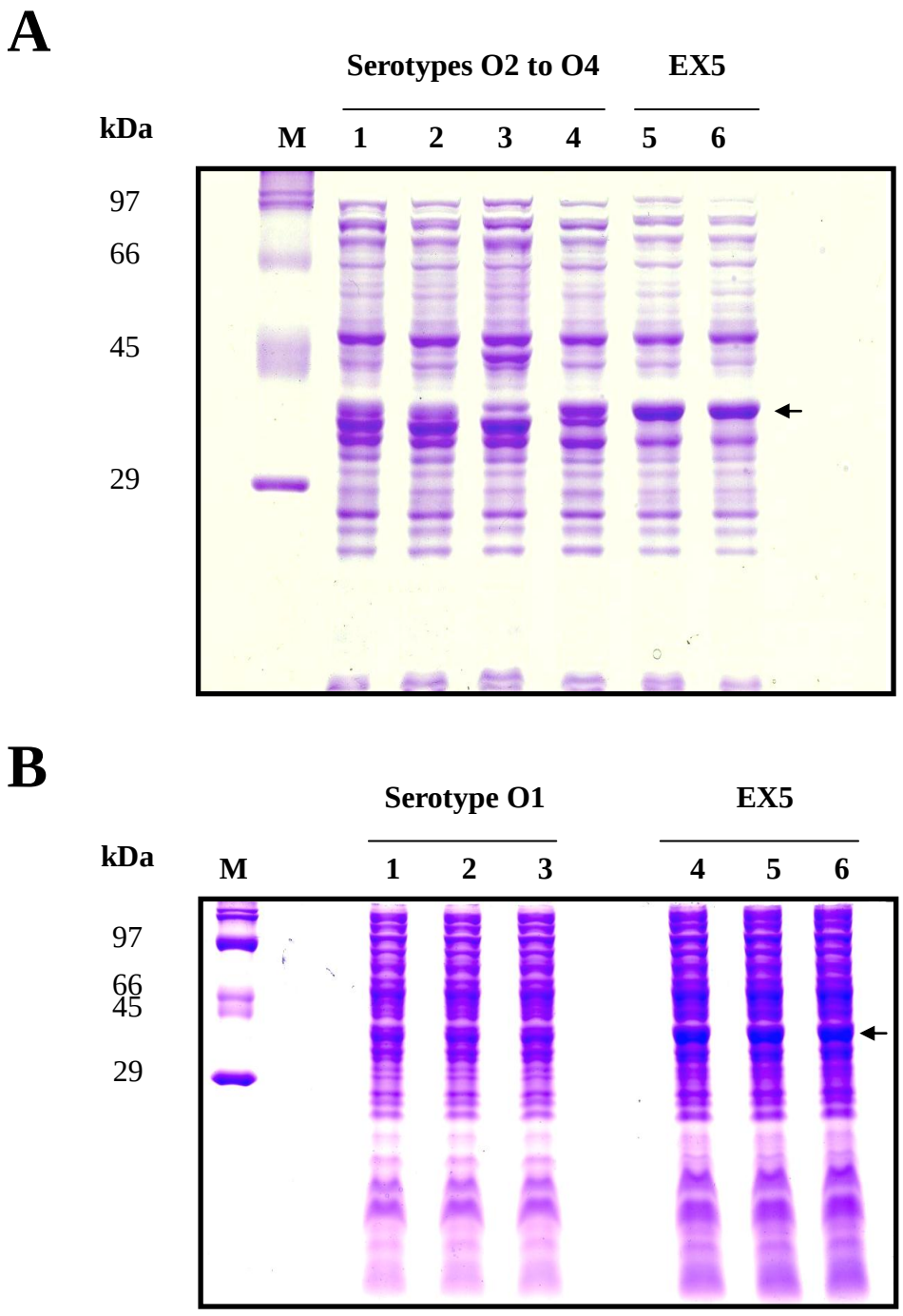


Fig 3. 24: Crude *Y. ruckeri* whole-cell proteins (WCP) representing different serotypes fractionated on a 10% (w/v) SDS-PAGE gel. Proteins were visualised by staining with Coomassie brilliant blue-R250. **A: Lanes 1 and 2:** O2BA2; **Lane 3:** O3BA3; **Lane 4:** O4BA4; **Lane 6:** EX5; **Lane 7:** EX5 (R1). **B: Lanes 1 to 3:** YR1; **Lanes 4 to 6:** EX5 (R1).

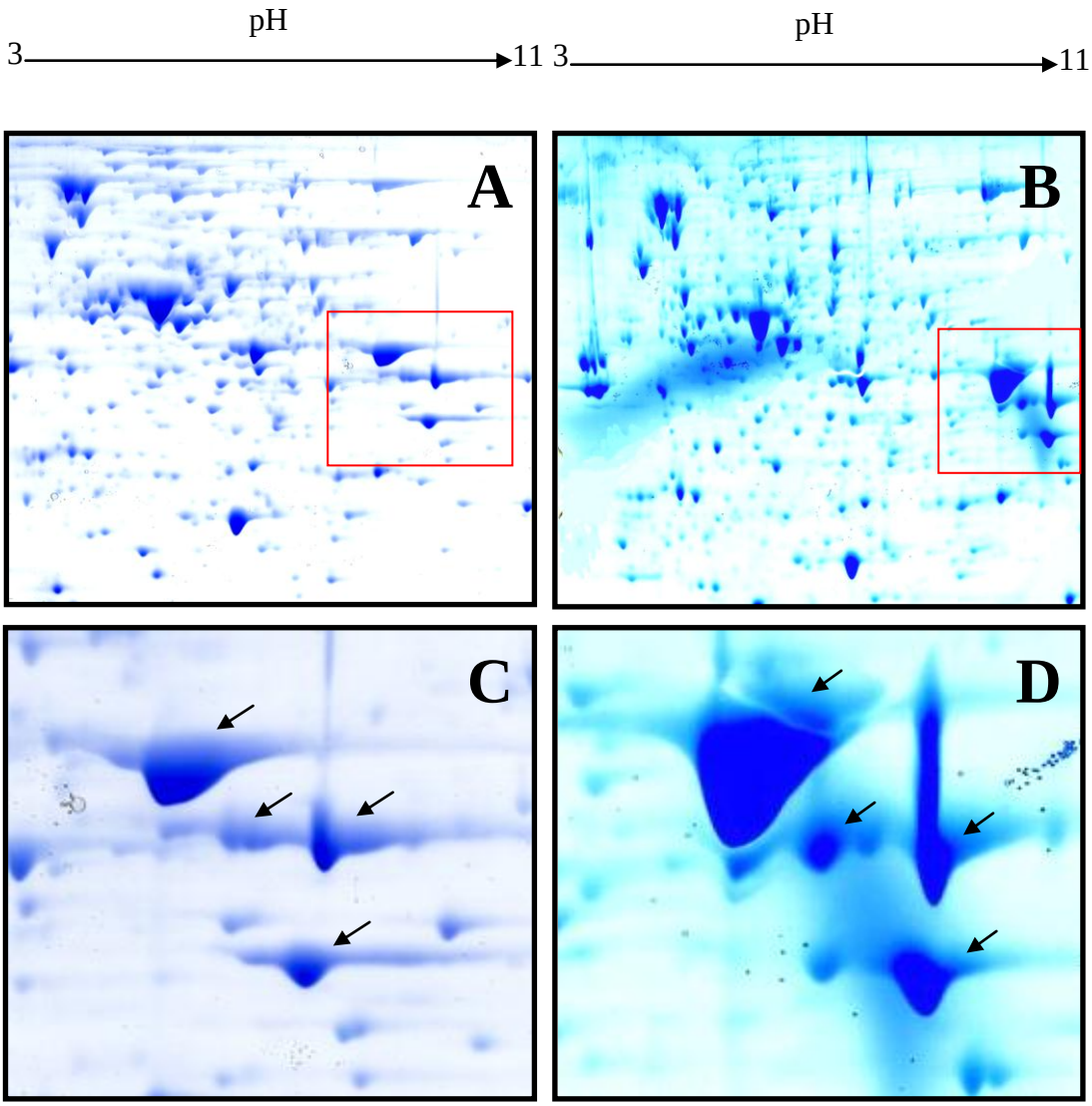


Fig 3. 25: Separation of *Y. ruckeri* serotype O1 (YR1) (**A**) and EX5 (R1) (**B**) WCPs on a large (24 cm) 2D (pH 3-11 NL) 10% (w/v) SDS-PAGE. Proteins were stained with colloidal Coomassie blue-G250. Differentially expressed proteins 30 to 40 kDa in weight are shown both for the serotype O1 (**C**) and EX5 (**D**) strain, which represent the enlarged insert boxes in **A** and **B**, respectively. These proteins are predominately more abundant within EX5 (**D**) compared to the serotype O1 strain (**C**).

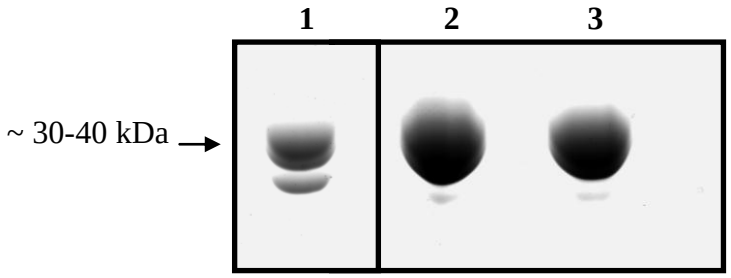


Fig 3.26: Sarkosyl purified outer membrane proteins (OMPs) from a *Y. ruckeri* serotype O1 (YR1) and EX5 (R1) isolate fractionated on a 10% (w/v) SDS-PAGE.

3. 18 Identification of the *Y. ruckeri ompA* gene

Given that EX5 isolates are overexpressing OmpA, attempts were made to identify the *Y. ruckeri* gene (*ompA*) encoding this protein. Again this initially posed a problem as the *Y. ruckeri* genome has not yet been sequenced. However, approaching this problem in a similar manner used to isolate the *Y. ruckeri* flagellin gene, it was found that both the N- and C- terminals of the *Y. enterocolitica ompA* gene were conserved among *Yersinia* spp. Hence the gene of interest was successfully amplified (Fig 3.27) using forward (OMPA-F) and reverse (OMPA-R) primers based on the *Y. enterocolitica ompA* gene sequence.

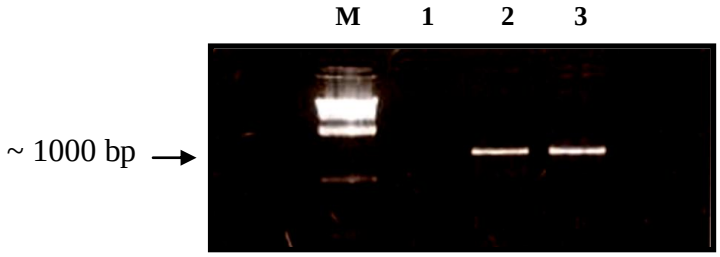


Fig 3.27: PCR amplification of the *Y. ruckeri ompA* gene using conserved forward (OMPA-F) and reverse (OMPA-R) primers. **M:** Molecular weight marker (λ*Hin*dIII). **Lane 1:** Negative control (H_2O as template). **Lane 2:** Serotype O1 (YR1) genomic DNA used as template. **Lane 3:** EX5 (R1) genomic DNA used as template.

The full *Y. ruckeri ompA* gene was sequenced and is available in the appendix (Fig 5.8) along with the predicted amino-acid sequence of the OmpA protein. Both the serotype O1 (YR1) and EX5 (R1) *ompA* ORFS were sequenced and found to be identical (100% identity). The *Y. ruckeri ompA* gene (1148 bp; 50.3% G+C) is predicted to encode a 34.08 kDa protein comprised of 321 amino acids. BLAST analysis of the predicted protein sequence showed that it shared a great deal of identity (84%) to the OmpA protein of *Y. pseudotuberculosis* strain IP 32953 (NCBI Reference Sequence: YP_069984.1) (Fig 3.28).

```
1    MKKTAITHSSALAGFATVAQAAPKDNTWYTGGKLGWSQFHDVGTGSDISNDGPTHKSQLG  60
1    MKKTAIALAVALVGFATVAQAAPKDNTWYTGGKLGWSQYQD--TGSIINNDGPTHKDQLG  58

61   AGAFVGYQANQYLGFEMGYDWLGRMPYKGDTVNGAFKAQGVQLAAKLSYPIAQDLDLYTR  120
59   AGAFFGYQANQYLGFEMGYDWLGRMPYKGDINNGAFKAQGVQLAAKLSYPVAQDLDVYTR  118

121  LGGMVWRADASVNEPSTNSHASAHDTGVSPLAAVGLEYAVTKNWATRLDYQWVNNIGDRG  180
119  LGGLVWRADAKGSFDGGLDRASGHDTGVSPLVALGAEYAWTKNWATRMEYQWVNNIGDRE  178

181  TVGARPDNGMLSVGVSYRFGQDDAIVPVVAPAPAPAPVVDTKRFTLKSDVLFAFNKATLK  240
179  TVGARPDNGLLSVGVSYRFGQEDAAAPIVAPTPAPAPIVDTKRFTLKSDVLFGFNKANLK  238

241  PEGQQALDQLYSQLSSIDPKDGSVVVLGFADRIGQAAPNLKLSENRARSVVEYLVAKGIP  300
239  PEGQQALDQLYAQLSSIDPKDGSVVVLGFADRIGQPAPNLALSQRRADSVRDYLVSKGIP  298

301  ADKISARGMGQADPVTGNTC  320
299  ADKITARGEGQANPVTGNTC  318
```

Fig 3.28: Alignment of the predicted amino-acid sequence of the *Y. ruckeri* OmpA protein with that of the OmpA protein from *Y. pseudotuberculosis* (IP 32953). The amino acid sequence of the *Y. ruckeri* OmpA protein is shown in bold. Conserved areas shared by the two proteins are highlighted in grey.

3.19 OmpA overexpression and gene copy number

As the *ompA* genes of *Y. ruckeri* EX5 (R1) and serotype O1 (YR1) are identical, this cannot account for why it is overexpressed in the EX5 (R1) strain. However, should the EX5 isolate have more than one copy of the gene, then this may explain as to why it is overexpressed. To determine copy number, a Southern blot was performed with *Y. ruckeri* genomic DNA before hybridizing with a DIG-labelled *ompA* probe. Briefly, genomic DNA from the serotype O1 (YR1) and EX5 (R1) strain was

purified and digested overnight with either *Eco*RI, *Hin*dIII or *Bam*HI before separating on a 0.7% (w/v) agarose gel (Fig 3.29). It is worth noting that these restriction enzymes used to digest DNA do not cut within the *ompA* gene itself. Digested genomic DNA was blotted to a membrane before hybridizing with the DIG-labelled *ompA* probe (Fig 3.29; B).

From these results it is clear that both the *Y. ruckeri* EX5 (R1) and serotype O1 (YR1) carry one single copy of this gene within the genome as only a single hybridization band is observable for each digest. It also shows that there are no gross alterations (for example a transposon insertion) to the genome at this locus, since the fragment size was identical in both genotypes. This would suggest that expression is regulated at the transcriptional level as opposed to an increasing in the number of *ompA* gene copies.

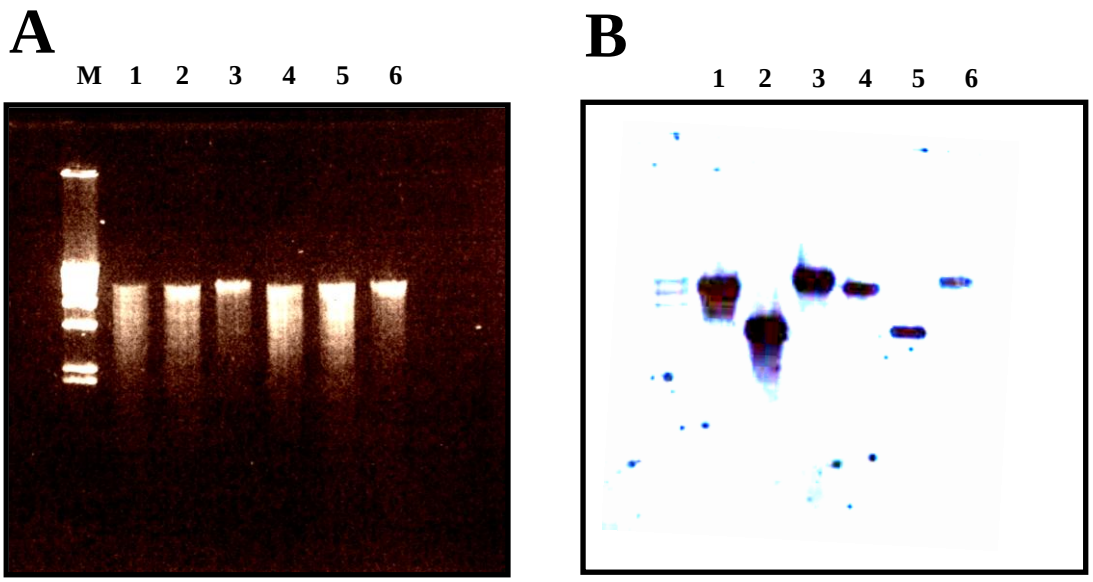


Fig 3.29: Detection of the *ompA* gene within the genomes of a serotype O1 (YR1) or EX5 (R1) *Y. ruckeri* strain by Southern blotting and hybridization/detection using a DIG-labelled *ompA* probe. **A:** Digested genomic DNA separated on a 1% (w/v) agarose gel. **B:** Hybridization/detection of *ompA* genes carried within the *Y. ruckeri* genome. **M:** Molecular weight marker (λ *Hin*dIII). **Lanes 1 to 3:** Genomic DNA from the serotype O1 (YR1) strain. **Lanes 4 to 6:** DNA from the EX5 (R1) isolate. DNA was digested with either *Bam*H1 (1 and 4), *Eco*R1 (2 and 5) or *Hin*dIII (3 and 6).

3.20 Screening for an *ompA*::Tn-RL27 *Y. ruckeri* EX5 (R1) transconjugant

Since large amounts of OmpA was seen to be produced by the *Y. ruckeri* EX5 (R1) strain, it is possible that this protein may contribute to virulence. Attempts were therefore made to disrupt the *ompA* gene encoding this protein (OmpA) with an aim of determining what role this molecule may have in virulence towards rainbow trout. Although a number of options are available for introducing mutations within a given gene, the Tn-RL27 transposon mutagenesis system described in Section 3.3 was chosen as it was shown to be efficient in generating large amounts of randomly integrated Tn-RL27 EX5 (R1) transconjugant mutants.

A fresh *Y. ruckeri* EX5 (R1) isolate, which was passed through naïve rainbow trout numerous times by Dr D. Austin (Heriot-Watt University) to confirm virulence, was conjugated with the *E. coli* BW20767/pRL27 strain. In total, 5 conjugations were performed using the *Y. ruckeri* EX5 (R1) and *E. coli* BW20767/pRL27 strains before plating bacteria out onto TSA (+ 50 µg/ml kanamycin, + 5 µg/ml nitrofurantoin) and incubating overnight at 28˚C. The conjugation was considered to be successful insofar as 20 randomly chosen colonies were all confirmed to be *Y. ruckeri* EX5 (R1). Moreover, a PCR reaction on all 20 colonies confirmed the presence of the gene encoding kanamycin resistance, thus showing that the Tn-RL27 transposon had successfully integrated within the genome. Successful transconjugation rates were not dissimilar to those obtained previously (Section 3.3).

From 100 TSA selection plates, each carrying ~100 colonies, 4800 were picked and grown in individual wells of a 96-well microtitre plate containing 200 µl TSB (+ 50 µg/ ml kanamycin, + 5 µg/ml nitrofurantoin). All mutants had grown after the 48 h incubation period.

Unlike the previous experiment, which used phenotypes to isolate the mutant of interest (Section 3.3), screening for *ompA* mutants could not make use of this method. However, the *ompA* forward (OMPA-F) and reverse primers (OMPA-R) were available, as were the flanking transposon primers TPNRL17-1 and TPNRL13-2. In principle, transconjugants with a Tn-RL27 transposon within the *ompA* gene (*ompA*::Tn-RL27) could be identified with PCR using a combination of these primers. A diagrammatic representation of this process can be seen in Fig 2.2. PCR

screening would also allow multiple samples to be tested at the same time by pooling data and using a high number of PCR cycles.

In total, samples from 50 microtitre plates (= 4,800 mutants) were pooled (100 µl from each well) in groups of 2 microtitre plates (= 192 mutants per group). Plates were then stored at -70˚C after the addition of glycerol. Genomic DNA was purified from the pooled cultures to give 25 samples. Thus, all 4,800 mutants were represented by 25 DNA samples.

Theoretically, the transposon could have inserted within the *ompA* gene in two orientations. This can be accommodated using different combinations of primers. Thus the *ompA* forward (OMPA-F) or reverse (OMPA-R) primer could be used in combination with the transposon flanking primers TPNRL17-1 or TPNRL13-2. This equates to a total of 4 PCR reactions for each pooled DNA sample, making 100 reactions in total for all 25 reactions.

Results from all initial PCR reactions are shown in Fig 3.30. The PCR products would have to be smaller (< 1000 bp) than the WT *ompA* gene for the transposon to be inserted within the *ompA* gene. Although PCR products were visible, none of these bands hybridized with the DIG-labelled *ompA* probe after Southern blotting. In this case, all PCR products were probably a result of non-specific primer binding. PCR conditions used here had an annealing temperature of 55ºC and 30 cycles. The experiment was repeated using an annealing temperature of 53ºC and 35 cycles. In total, six separate DIG probe hybridising bands were detected using the OMPA-F and TPNRL13-1 primer (not shown), each smaller than the WT *ompA* gene. This would suggest that the Tn-RL27 transposon had inserted within the gene of interest (*ompA*). Unfortunately, this was later found to be a result of non-specific primer binding as 50 colony PCR reactions on individual transconjugants all hybridized with the *ompA* probe. Moreover, decreasing the number of PCR cycles (34 to 28) and increasing the annealing temperature (53 to 55ºC) for a second round of colony PCR did not result in any PCR products or hybridized bands. At this point in the project it seemed unlikely that the transposon had inserted within the *ompA* gene of any *Y. ruckeri* transconjugant.

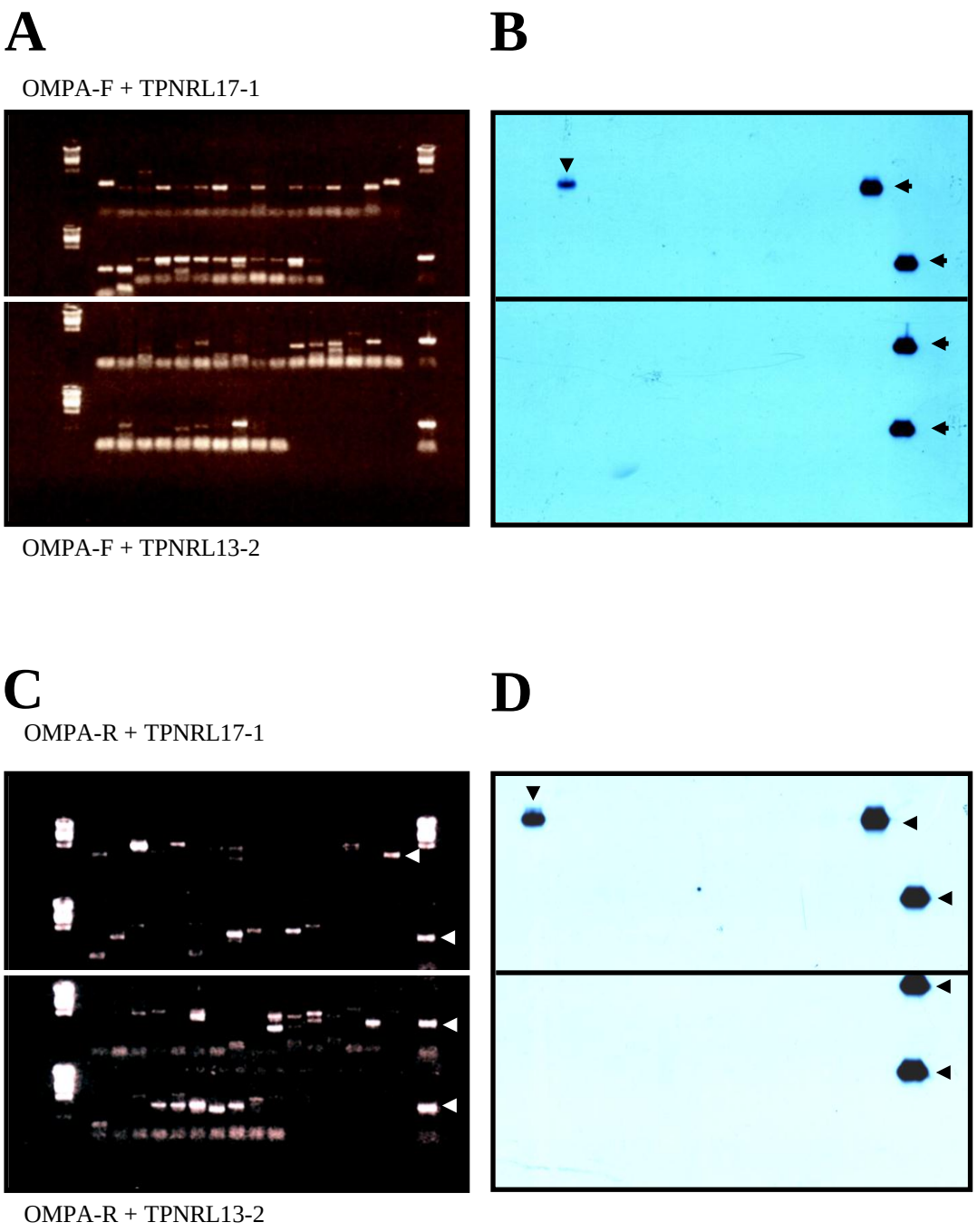


Fig 3.30: Example of PCR screening and Southern blotting to detect for a *Y. ruckeri* EX5 (R1) transconjugant with a transposon within the *ompA* gene (*ompA*::Tn-RL27). PCR was performed at 55ºC for 30 cycles with different primer combinations before separating on a 1% (w/v) agarose gel (A and C) and blotting/hybridizing with the DIG-labelled *ompA* probe (B and D). PCR reactions were performed with the primer OMPA-F (A) with TPNRL17-1 (1) or TPNRL13-2 (2). Reactions were also carried out with the OMPA-R (C) and TPNRL17-1 (3) or TPNRL13-3 (4) primer. Whereas all positive PCR *ompA* products (generated using

the OMPA-F and OMPA-R primers) hybridized with the *ompA* probe (indicated), no transconjugant strains were detected using the DIG-labelled primer.

CHAPTER 4: DISCUSSION

4.1 General discussion

Aquaculture is currently the fastest growing sector of agriculture in many countries, with the notable exception of Sub-Saharan Africa (Plant and Lapatra, 2011), and has increased its annual production of fish from 28 to 50 million tonnes between 1998 and 2007 (National Oceanic and Atmospheric Administration, 2007). Unfortunately, as production of fish increases, so does the incidence of disease. Currently, disease is the major contributor to the loss of fish in aquaculture. In fact 90% of all the deaths associated with rainbow trout reared in captivity in 2009 were considered to be due to disease (National Agricultural Statistic Service, 2010). This can often have significant socio-economic consequences, particularly in rural areas and/or developing countries. Knowing the impact that disease can have for the industry, controlling and preventing disease is of paramount importance.

ERM is an example of a problematic disease in aquaculture, with much effort recently being expended in an attempt to develop effective control strategies. Preventing ERM was initially a success story insofar as the early application of an inactivated whole-cell immersion vaccine successfully prevented major disease outbreaks (Austin, 2007). Actually, this was the first bacterial fish vaccine to be officially licensed by the USA in 1976 for use in aquaculture (Plant and LaPatra, 2011). However the success of the original vaccine led to a decline in research aimed at other methods of disease control, or indeed the disease process itself. During the early 1980s, most of the research regarding ERM was based around the nature of the fish immune system and its response towards live or inactivated *Y. ruckeri* vaccines (Austin, 2007). It was not until the early 1990s that research started to focus on the molecular basis of pathogenicity (Romalde and Toranzo, 1993, Davies, 1991a, Davies, 1991b, Romalde *et al*., 1991, Furones *et al*., 1990). The last decade alone has seen a significant increase in the number of publications regarding this fish pathogen. This upturn in research is due to a number of reasons. Firstly, antimicrobial compounds are no longer as effective in preventing disease due to an increase in bacterial resistance. Moreover their use in a non-urgent or prophylactic situation is also not encouraged because of the perceived effect on antibiotic resistance of human pathogens. Antimicrobials can also be a costly commodity, particularly if they have little or no effect in preventing/treating disease. Secondly,

the recent failure of the monovalent whole-cell vaccine in protecting against *Y. ruckeri* has again stimulated interest in this fish pathogen (Austin *et al*., 2003). Finally, with the advent of sophisticated molecular biology techniques, gaining an insight into the molecular basis of *Y. ruckeri* pathogenicity is now becoming possible (Tobback *et al*., 2007, Fernandez *et al*., 2007).

4.2 ERM and a new problem for aquaculture

Y. ruckeri strains which are capable of causing disease in monovalent-vaccinated livestock shared a number of phenotypic and biochemical features. One notable phenotype observed by Austin *et al.* (2003) regarding these isolates was their apparent lack of motility and Tween 20/80 degradation; phenotypes which were not usually associated with *Y. ruckeri*. Other authors have since isolated similar non-motile strains from dead and/or moribund fish (Arias *et al*., 2007, Davies and Frerichs, 1989, Strom-Bestor *et al*., 2010, Fouz et al., 2006, Wheeler *et al*., 2009, Evenhuis *et al*., 2009, Welch *et al*., 2011, Welch, 2011). It is probable that these *Y. ruckeri* strains belong to the serotype O1 (= serovar I) subgroup as they are unable to ferment sorbitol and have an increased virulence towards rainbow trout. Moreover, LPS from these non-motile strains hybridized with rabbit polyclonal antibodies against serotype O1 LPS (Fouz *et al.*, 2006). Serotype O1 isolates are also associated with the presence of a 50 MDa plasmid (Garcia *et al*., 1998), the presence of which was also confirmed to be within non-motile isolates obtained by Austin *et al.* (2003) in this study (not shown). Thus it is likely that non-motile strains, termed EX5, were originally serotype O1 isolates which have naturally caused disease in rainbow trout previously vaccinated against ERM.

4.3 EX5 and loss of flagellin expression

Since the initial isolation of non-motile EX5 isolates, there has been considerable interest regarding the non-motile phenotype, particularly since the majority of ERM outbreaks were previously associated with serotype O1 (= motile) *Y. ruckeri* strains. In addition to being non-motile, Evenhuis *et al.* (2009) showed that the flagellin protein was not produced by EX5 isolates *in vitro* under standard culturing conditions, i.e. 72 h at 15°C in TSB. This was demonstrated using a monoclonal antibody described by Feng *et al.* (1990) which recognises a conserved epitope present on Enterobacteriaceae flagellin. It is noteworthy that while Feng *et al.* (1990) found that that this antibody reacted specifically with Enterobacteriaceae

flagellin, it is likely that the sequence recognised by this antibody is within the N- or C- terminal as these regions are conserved amongst this group of bacteria (Fig 3.13). Work presented in this thesis using the monoclonal antibody raised in mice against flagellin by Feng *et al.* (1990) confirms observations by Evenhuis *et al.* (2009) as to the lack of flagellin production in EX5 isolates (Fig 3.4). Culturing EX5 under conditions which are known to influence virulence (i.e. iron and temperature limiting) again had no effect upon the non-motile phenotype, nor was flagellin detectable using the anti-flagellin monoclonal antibody (Fig 3.23). Similarly, passing non-motile *Y. ruckeri* isolates through naïve fish again had no effect on motility or flagellin production.

Using conserved primers for flagellin, it was shown by PCR that *Y. ruckeri* EX5 strains do carry the gene encoding flagellin (*fliC*). Similarly, Evenhuis *et al.* (2009) found that the genes involved in flagellar secretion, organised as an operon (termed *flhBAE-flgNMABC*), are also carried within the genome of non-motile *Y. ruckeri* isolates. Interestingly, the *fliC* gene was not within the *flhBAE-flgNMABC* operon, suggesting that it is transcribed separately. The presence of flagellin (*fliC*) or flagellin-associated genes within the EX5 genome indicates that these isolates have previously arisen from a motile ancestor.

One explanation for the lack of flagellin production is that the EX5 strain has acquired mutations in genes required for flagellin production. Evenhuis *et al.* (2009) showed that inactivating the putative flagellin transport operon (*flhBAE-flgNMABC*) via transposon mutagenesis completely repressed flagellin production for a motile *Y. ruckeri* serotype O1 isolate. Functional complementation with the wild-type *flhBAE-flgNMABC* operon reversed the non-motile phenotype. Hence, introducing a mutation within a gene associated with flagellin and/or flagellar assembly can completely repress the production of the flagellin protein, even if the mutation is not within the *fliC* gene itself. Results from transposon mutagenesis experiments carried out in this study confirm this observation; transposon insertions within motility-associated genes can completely repress flagellin production. However the mechanism by which flagellin production is repressed in *Y. ruckeri* is currently unknown. Evenhuis *et al.* (2009) suggested that it may not be dissimilar to *Salmonella enterica* serovar Typhimurium whereby the expression of flagellin is dependent on the successful completion of the flagellin basal-hook body (Rosu and

Hughes, 2006). In this bacterium the unnecessary expression of flagellin is prevented when other necessary components of the flagellar architecture (i.e. basal hook proteins) are not present or produced by the bacterial cell.

Whereas Evenhuis *et al.* (2009) found that inactivating flagellin-associated genes results in the complete repression of flagellin production, they did not describe the specific genes which have been rendered non-functional in naturally occurring EX5 isolates. However, Welch *et al.* (2011) discovered that the natural mutations responsible for causing loss of flagellin production in EX5 strains have all arisen within the *fliPQR* gene cluster; a group of genes thought to encode integral membrane components of the flagellar secretion pathway. All UK and US EX5 strains employed by Welch *et al.* (2011) carried a *fliR*Δ1 mutant allele caused by the deletion of a single nucleotide within the *fliR* gene at codon 30 of 260. This links well with the view that non-motile strains in the USA and UK are clonal (Tinsley et al., 2011a, Wheeler et al., 2009). Using functional complementation, it was possible to convert a non-motile (= flagellin devoid) EX5 isolate back to a motile (= flagellin producing) phenotype by complementing with the WT *fliR* gene. This confirms the previous hypothesis by Evenhuis *et al.* (2009) which states that the expression of flagellin is dependent on the successful completion of underlying membrane proteins involved in the flagellar architecture (Rosu and Hughes, 2006). Also, it shows that the *fliC* gene in EX5 is not mutated as a functional flagellin can still be produced when complemented with the WT *fliR* gene. Taking results outlined in both this work and that of others (Evenhuis et al., 2009, Welch et al., 2011), EX5 isolates are unlikely to be producing flagellin or even a truncated/undetectable form of the protein under any condition.

4.4 EX5 and flagellin loss: Immune evasion?

As EX5 strains cause disease in vaccinated fish, it seemed possible that some form of Darwinian selection pressure has driven the appearance of the non-motile phenotype. However, the nature of this pressure has not yet been identified. It is understood that the expression of flagellin within the mammalian host can be a potent initiator of the innate immune system, thus its repression and/or loss could confer a selective advantage for a pathogenic bacterium (Minnich and Rohde, 2007, Steiner, 2007). For example, *Y. pestis* was originally motile, although it was rendered non-motile due to the acquisition of mutations within the *fliD* gene

(Minnich and Rohde, 2007). This is thought to have resulted in an increase in virulence towards mammals as the loss of flagellin production presumably prevented the detection/stimulation of an innate immune response. Indeed, as was the case with *Y. enterocolitica*, the artificial expression of flagellin within the host can completely attenuate virulence (Minnich and Rohde, 2007).

Since EX5 isolates do not produce detectable flagellin, it was reasonable to consider whether the loss of flagellin production aided in bacterial evasion of the fish immune system. However, Evenhuis *et al.* (2009) showed that the mutational loss of flagellin production in a motile serotype O1 *Y. ruckeri* background (discussed earlier) did not increase pathogenicity towards naïve rainbow trout. Similarly, Wiens and Vallejo (2010) demonstrated that challenge with an EX5 strain still resulted in a significant up-regulation of innate immunity (e.g. TNFα1, IL1-β1 and SAA). Hence the presence/absence of flagellin does not affect the robust pro-inflammatory and acute-phase gene transcription response towards EX5 infection. Moreover, this work has shown that inactivated EX5 cells can be used as an effective vaccine against challenge with a serotype O1 (YR1) strain; hence EX5 may still stimulate an immune response even in the absence of flagellin. Overall, taking data by Evenhuis *et al.* (2009) and Wiens and Vallejo (2010) into consideration, it would seem unlikely that EX5 strains can evade immune recognition/stimulation via the mutational loss of flagellin.

4.5 Flagellin as a component of a vaccine

Assuming that the loss of flagellin expression by EX5 isolates does not directly influence virulence for naïve rainbow trout, it remains perplexing why there was a sudden increase in the number of ERM outbreaks associated with the non-motile phenotype, particularly when the majority of previous cases were linked with motile strains. Furthermore, ERM outbreaks associated with the non-motile phenotype occurred in livestock which had previously undergone vaccination against this disease (Austin *et al.*, 2003). As noted by Tobback *et al.* (2007), it is strange why vaccination with the monovalent vaccine (= inactivated serotype O1 *Y. ruckeri* cells) did not protect against the EX5 strain, particularly since Cipriano and Ruppenthal (1987) found that vaccinating fish with serotype O1 cells can broadly protect against other serotypes. However, understanding why the vaccine failed to protect may be

problematic, particularly since the main molecules involved in eliciting a protective immune response towards *Y. ruckeri* are poorly understood (Tobback *et al*., 2007).

One possible theory, which has recently been proposed by numerous authors in the published literature (Tobback *et al*., 2007, Evenhuis *et al*., 2009, Welch *et al*., 2011, Wheeler *et al*., 2009), is that prolonged and extensive vaccination against a serotype O1 strain has exerted a selection pressure on the motile (= flagellin-producing) phenotype. Thus, should flagellin and/or flagellin associated molecules be an important component of the monovalent vaccine for conferring protection against a serotype O1 strain, then EX5 cells may be able to evade a vaccine-mediated response directed towards flagellin. This would explain the link between vaccine failure and the non-motile phenotype.

To address sufficiently the immunoprotective/immunostimulatory role of flagellin as part of a whole-cell vaccine, all possible antigens and/or immunostimulatory molecules aside from flagellin (including flagellin-associated molecules) would have to be removed from the vaccine preparation. On the other hand, potentially immunogenic/immunostimulatory molecules not associated with flagellin (e.g. OMPs and LPS) would presumably have to remain. Like Evenhuis *et al*. (2009), a flagellin-devoid serotype O1 strain was obtained using the transposon mutagenesis system described by Larsen *et al*. (2002). Sequencing DNA flanking the transposon insert found that the transposon had inserted within the *flhA* gene of the *flhBAE-flgNMABC* operon, a region of DNA which was also disrupted by transposon mutagenesis in the research carried out by Evenhuis *et al*. (2009). It seemed unfortunate that the transposon had inserted within the same region, particularly since it may have elucidated the roles of other genes involved in motility and/or flagellar biosynthesis. The fact that the transposon has inserted within this site on two different occasions suggests that this region of genomic DNA is a so-called "hot-spot" for transposon insertions. Nonetheless, a flagellin-devoid transconjugant was adequate for the purposes of this study.

Assuming that extensive vaccination against a flagellin-producing serotype O1 *Y. ruckeri* strain resulted in a rise in the number of ERM cases associated with flagellin-devoid EX5 strains, fish vaccinated with serotype O1 would presumably be protected against challenge with a serotype O1 strain, yet susceptible to infection

with an EX5 *Y. ruckeri* isolate. This possibility was addressed here by vaccinating with the serotype O1 (BA19) parent (= flagellin-producing) and non-motile BA19/Tn-RL27 (= flagellin-devoid) mutant strain. Vaccinates were then challenged with the serotype O1 (YR1) (= flagellin-producing) or EX5 (R1) (= flagellin-devoid) strain.

For the purposes of this study, vaccine efficiencies were evaluated by calculating the relevant per cent survival (RPS) after 2-weeks post challenge. Using these criteria, it was found that vaccinating fish with the serotype O1 (BA19) isolate conferred moderate levels of protection against challenge to a serotype O1 (YR1) strain (RPS = 65%), but not to an EX5 (R1) isolate (RPS = 23%). This result represents the inefficiency of the monovalent (= serotype O1) vaccine to effectively protect against the EX5 strain. However, when flagellin was essentially removed from the vaccine preparation using the BA19/Tn-RL27 mutant strain, levels of protection towards challenge with the serotype O1 (YR1) fell only slightly (total survival from 76% to 60%). This decrease in the levels of survival following bacterial challenge is not statistically significant ($P \leq 0.05$; $N = 25$), suggesting that flagellin is not the main immunostimulatory molecule in eliciting a protective immune response towards infection. Furthermore, removing flagellin from the vaccine also resulted in a small reduction in the level of protection against the EX5 (R1) strain (total survival from 60% to 46%). Although this is not statistically significant ($P \leq 0.05$, $N = 25$), it does indicate that flagellin may have a minor role in eliciting a non-specific immune response towards a flagellin-devoid strain in a serotype O1 background. Increasing the number of replicates in each group (i.e. 25 to 250) may show that flagellin, although not entirely required for protection, can nonetheless contribute towards a non-specific immune response.

In addition to vaccinating fish with the serotype O1 (BA19) and BA19/Tn-RL27 mutant, fish were also vaccinated with inactivated EX5 cells. When used as a vaccine, EX5 conferred excellent levels of protection (RPS = 95%) towards challenge with the serotype O1 (YR1) strain. This shows that flagellin is not the main molecule responsible for mounting a protective immune response since EX5, which does not produce flagellin, can effectively protect against a flagellin-producing strain.

Thus from these experiments it would seem unlikely that flagellin is the main immunogenic/immunostimulatory molecule in the vaccine leading towards protection against a serotype O1 strain. It also seems unlikely that extensive vaccination has led to the development and spread of non-motile isolates. Nonetheless, *Y. ruckeri* must be producing other kinds of immunostimulatory/immunogenic molecules since the EX5 isolate can protect against challenge with the serotype O1 (YR1) strain. Tinsley *et al*. (2011b) recently suggested that the O-antigen of LPS is the main immunogenic molecule in conferring protection against ERM. This is entirely possible since LPS, in itself a well-established PAMP, can be a powerful immunogen for fish (Austin, 2007). However, it is likely that a combination of molecules in addition to LPS is contributing to an effective immune response, although this would require further research.

Due to time constraints and limited space in the aquarium, challenges were performed 28 days post-vaccination. Whilst this time period is generally sufficient for evaluating the effectiveness of a given vaccine, it does have some limitations insofar as a humoral response (i.e. antibody production) will not generally occur until after 8 or 12 weeks post-vaccination (Raida et al., 2011). Although the role of antibody-mediated protection in salmonids towards *Y. ruckeri* is still a contentious area of debate, Welch *et al*. (2011) suggested that flagellin may be the main immunogenic molecule in the original vaccine. Thus long term vaccination against a serotype O1 *Y. ruckeri* strain has led to the production of antibodies which specifically target flagellin and/or flagellin molecules. In this respect, vaccinates would presumably be susceptible to infection with EX5 since they do not produce flagellar antigens. However, this would be unlikely for a number of reasons. On the one hand, IgM produced by fish towards inactivated bacteria would presumably be polyclonal and thus react against a whole range of antigenic determinants (e.g. LPS and OMPs). If flagellin was absent, then polyclonal antibodies would theoretically still agglutinate the bacterium. Secondly, a recent study by Deschmuch *et al*. (2012) showed that vaccinating fish with a serotype O1 strain did in fact offer some level of cross-protection against challenge with an EX5 isolate 8 or 12 weeks post-vaccination. Therefore, long-term protection must not be entirely dependent on anti-flagellin antibodies. Finally, the inadequacy of the serotype O1 vaccine to protect against challenge with the EX5 strain is observable after 1 month post-vaccination (Fig 3.6). As discussed, antibody production is unlikely to have occurred within this

limited time period (Raida *et al.*, 2011). This shows that the serotype O1 vaccine still fails to protect sufficiently against the EX5 strain even in the absence of antibody production. Overall, it would seem unlikely that vaccination against a flagellin-producing serotype O1 isolate has resulted in the non-motile phenotype of EX5 strains.

4.6 Reasons for the non-motile phenotype of EX5

The majority of research to date regarding EX5 strains has focused on the non-motile phenotype of EX5 isolates, principally in an attempt to link loss of motility with an increase in virulence. As previously discussed, it initially seemed likely that EX5 strains were evading the immune system via the loss of flagellin production *in vivo*. However, this theory has largely since been disproven since EX5 cells can still stimulate an immune response even in the absence of flagellin. Others have since suggested that excessive vaccination against a motile strain has resulted in the non-motile phenotype; experiments outlined in this study suggest otherwise.

Why then is there a sudden increase in ERM cases associated with non-motile EX5 strains? It is reasonable to assume that flagellar production was previously required for spread and infection since serotype O1 isolates are motile. However, it is possible that the rapid expansion and intensification of aquaculture has led to a rise in the non-motile phenotype. For example, non-motile bacteria could presumably be spread in faecal material which would, in turn, come into contact and/or be ingested by neighbouring fish. Dead carcases infected with EX5 cells may also be a recurring source of infection, particularly as fish are known to be cannibalistic. As to bacterial survival in the natural environment, *Y. ruckeri* may remain viable in faecal material for a number of months (Hunter *et al.*, 1980, Rodgers, 1992), perhaps even longer in a non-culturable state (Romalde *et al.*, 1994a) or as biofilms (Coquet *et al.*, 2002a, Coquet *et al.*, 2002b). Of note, Coquet *et al.* (2002) found that outer membrane proteins contributing to motility were overexpressed by serotype O1 cells involved in biofilms. It would be interesting to fully determine what effect the lack of flagellin production by EX5 has on the establishment of these structures. However, even if the formation of biofilms is dependent on flagellin production, it is of little consequence since EX5 can effectively cause disease and persist within the natural environment.

Spread of EX5 over long distances may be facilitated via the movement of asymptomatic livestock (Busch and Lingg, 1975). Studies to date have shown that fish stocked in overpopulated and unsanitary waters become stressed, thereby leading to the immunocompromised state. This would presumably aid in both the spread and establishment of ERM by non-motile EX5 cells. In fact, EX5 isolates were originally obtained from dead fish sourced from a severely overstocked and poorly managed fish farm. Clearly, the motile phenotype is not required for the spread of bacteria between hosts, which might have been facilitated by the intensification of aquaculture i.e. overstocking and poor management.

Questions have also been raised over how non-motile EX5 cells establish infection, primarily since flagella are known to play a major role in the attachment and invasion of bacteria for host tissues. A notable example is *V. anguillarum*, a bacterial fish pathogen which requires a functional flagellin protein to establish an infection (Milton *et al*., 1996). Nonetheless, other molecules such as LPS, pili and adhesins are known to contribute to attachment and adherence. Tobback *et al*. (2008) found that *Y. ruckeri* attachment to host cell tissues involves a number of macromolecules which are proteinaceous and/or carbohydrate in nature. Furthermore, researchers have shown that EX5 strains retain the ability to be adhesive and invasive for both gill and gut mucus (Tobback *et al*., 2010). The very fact that a flagellin-devoid serotype O1 mutant can cause disease in rainbow trout when challenged via submersion (fish are bathed in a bacterial suspension) demonstrates that flagellin is not an essential requirement for establishing an infection *in vivo* (Evenhuis *et al*., 2009). Thus while flagellin production might have a very minor role in attachment and/or invasion in a serotype O1 background, its loss is not detrimental for retaining infectivity or virulence by EX5 cells towards fish.

As flagellin is evidently not required for infectivity and virulence, its loss is not detrimental for EX5 bacterial cells. Similarly Welch *et al*. (2011) suggested that mutations in flagellar genes may represent a form of genomic decay. It could be theorised that EX5 strains acquiring mutations in flagellin-associated genes might have a selective advantage over traditional serotype O1 strains. Should EX5 conserve metabolic energy which would otherwise be expended constructing the complex flagellar architecture, then this free energy may be redirected towards other

aspects of virulence. It would be of interest to perform co-infection experiments with a motile and non-motile mutant to determine if non-motile strains have a selective advantage over flagellin producing isolates *in vivo*. If non-motile isolates do have a selective advantage over motile bacteria, then it may explain why there is an increase in the isolation of non-motile strains. However, it is also possible that the non-motile phenotype of EX5 strains has been a so-called "red herring" in that research to date has potentially ignored other aspects of EX5 pathogenicity.

4.7 Native flagellin as a sub-unit vaccine

In addition to investigating the protective role of flagellin as a component of a whole-cell vaccine, the use of native flagellin as a sub-unit vaccine was also investigated. This experiment was initially performed to investigate the protective properties of flagellin as part of a whole-cell vaccine. Results demonstrated that native flagellin purified using the acid disassociation and re-association method described by Ibrahim *et al*. (1990), when used as a vaccine for naïve rainbow trout by i.p. injection (50 µg/fish), can confer excellent protection (RPS = 100%) against challenge with a serotype O1 (YR1) or EX5 (R1) *Y. ruckeri* strain 28-days post-vaccination. This was an unexpected, yet potentially significant result for a number of reasons.

As previously discussed, it was proposed by some researchers that excessive vaccination towards a serotype O1 *Y. ruckeri* strain might have exerted a selection pressure on flagellin, particularly if it is the main immunogenic molecule in the monovalent vaccine. Theoretically, this would explain why EX5 strains can cause disease in the vaccinated host. Thus in addition to investigating the protective properties of flagellin as part of a whole-cell vaccine, experiments were carried out using flagellin as a sub-unit vaccine. In this respect, should flagellin be an essential component of the monovalent vaccine, then vaccinates administered with flagellin would be protected against the flagellin-producing serotype O1 isolate, yet susceptible to challenge with the flagellin-devoid EX5 strain. This was clearly not the case since flagellin protected against both. Results thereby show that flagellin could protect against a flagellin-producing and flagellin-devoid strain, implying that flagellin is a highly effective, non-specific sub-unit vaccine.

From experiments investigating flagellin as part of a whole-cell vaccine, it was concluded that flagellin is not a major component in conferring protection. On the other hand, the use of flagellin as a sub-unit vaccine sufficiently protected against bacterial challenge. These results might appear to be contradictory; however, this could simply be down to a matter of dosage. For example (assuming a recovery of 100%), approximately 5 mg of native flagellin from 6 L of bacterial culture (1×10^9 cells/ml) could be obtained. Thus, each inactivated whole-cell preparation (1×10^8 cells/fish) contains approximately 0.08 µg (83 ng) of flagellin per fish. On the other hand, fish vaccinated with native flagellin were administered with 50 µg/fish; effectively 625 times more flagellin than that present within the whole-cell vaccine. It would seem likely that flagellin, while still not the main molecule responsible for eliciting a protective immune response in the whole-cell serotype O1 vaccine, can effectively protect when administered to fish in sufficient quantities.

The non-specific nature of protection conferred by flagellin towards a strain which is not producing this molecule is significant for a number of reasons. Should a vaccine target a specific antigen and/or combination of antigens which are produced by a bacterium *in vivo*, then there is the risk that immunity generated by vaccination can break down through selection pressure on the bacterium. For example the extracellular protease Yrp1, when used as an inactivated toxoid, was shown to protect against a Yrp1-producing *Y. ruckeri* strain (termed Azo^+) (Fernandez *et al.*, 2003). However, while production of this enzyme may have a role in establishing disease for Azo^+ isolates, it is not an essential prerequisite for virulence since some highly pathogenic *Y. ruckeri* strains do not produce this protease (termed Azo^-) (Secades and Guijarro 1999). Consequently, extensive vaccination with the Yrp1 toxoid could result in a rise in ERM cases associated with Azo^- *Y. ruckeri* strains. Similarly, the theory that extensive vaccination against a flagellin-producing serotype O1 *Y. ruckeri* strain has resulted in the rise of flagellin-devoid EX5 isolates, although probably not the case, would be an example of this break-down in immunity.

Whereas other vaccines may exert a selective pressure on the pathogen, it is likely that the use of flagellin as a vaccine would not result in such a selection pressure since protection is not dependent upon the expression of flagellin by the invading pathogen e.g. EX5. This would make it difficult for the pathogen to adapt and/or

overcome such a potent, non-specific immune response. Since protection is non-specific, there is also the real possibility that protection is not confined to this bacterial pathogen. Certainly the non-specific nature of protection potentially points towards the use of flagellin as a broad-spectrum vaccine candidate for conferring protection against a variety of pathogens problematic in aquaculture.

Compared to inactivated whole-cell vaccines used in this study, RPS values for challenge against both a serotype O1 and EX5 strain was excellent (100%) when using flagellin as a sub-unit vaccine. This is particularly encouraging as even the most efficacious whole-cell or sub-unit *Y. ruckeri* vaccine does not give RPS values of ~100%. For example the current ERM vaccine described by Austin *et al.* (2003), which has been made commercially available to prevent serotype O1 and EX5 outbreaks, was shown to confer excellent protection against a serotype O1 strain (RPS = 97%). On the other hand, only moderate levels of protection against a fresh EX5 isolate was obtainable (RPS = 56%). Thus the use of flagellin as a sub-unit vaccine can offer better levels of protection 28-days post-vaccination against ERM compared to the vaccine currently available.

Overall, it would seem that flagellin is potentially an effective, non-specific sub-unit vaccine candidate against ERM. However, the acid disassociation and re-association method used to obtain native flagellin was labour intensive, costly and time consuming (e.g. 4 to 5 days) for comparatively low yields of protein (e.g. 5 mg). Hence while adequate for the purposes of small scale vaccination studies, this protein purification technique would not be economically viable on a large scale, nor for further experimentations.

4.8 Use of recombinant *Y. ruckeri* flagellin (r-flagellin) as a sub-unit vaccine

In recent years, recombinant DNA technology has been used to create live/attenuated bacteria for administering to fish. A prominent example of such a vaccine is the *aroA* auxotrophic *Y. ruckeri* mutant discussed in Section 1.17.2. However, sub-unit vaccines have also been developed using recombinant methods; a prime example being the VP2 protein coat particle of infectious pancreatic necrosis virus (IPNV) (Fridholm *et al.*, 2007). In this respect, large amount of antigen can be overexpressed in *E. coli* which can then be purified for administering to fish as a

vaccine. This technique also has the added benefit of being fast (recombinant protein is obtainable within 24 to 48 h) and is relatively cost/labour effective for manufacturing. As native *Y. ruckeri* flagellin was an effective sub-unit vaccine against ERM, yet limited the means of purification methods, it was deemed reasonable to investigate the possibility of flagellin as a recombinant vaccine.

As shown in the results (Section 3.9), the gene encoding *Y. ruckeri* flagellin (*fliC*) was successfully cloned and overexpressed in *E. coli*. Yields of flagellin were in the region of 40 mg/L; approx. 8 times higher than that obtainable from 6 L of bacterial culture using the acid disassociation and re-association method. Furthermore, protein could be easily expressed by growing cultures overnight at 28°C ($OD_{600} \geq 1.0$) and inducing with 0.1 mM IPTG for 4 h; thus simplifying production and reducing production costs. Proteins could also be purified from inclusion bodies or by passing through an IMAC column, thus removing and/or limiting contamination with other molecules (e.g. LPS). Overall, the recombinant method of producing flagellin is far more effective and thus makes it appealing for use in production on a commercial scale.

Like native flagellin, administering r-flagellin to naïve fish protected against a virulent serotype O1 and EX5 *Y. ruckeri* strain. Challenging fish initially posed a problem in that the virulence of bacterial cultures varied significantly. For example, a dose held to be 1×10^5 EX5 cells/fish killed the majority ($\geq$ 90%) of control fish within 48 h. This was probably due to a miscalculation in bacterial numbers. Nonetheless, it does show that flagellin is effective against ERM as even the most efficacious vaccine and/or immune host would not be able to protect against such a high inoculum. Another interesting observation was that protection conferred by flagellin in this case was dose dependent. However, reducing the bacterial load used to challenge fish demonstrated that comparatively low amounts of this protein (e.g. 10 µg/fish) can offer significant protection against disease.

In addition to protecting against ERM-associated mortalities, administering flagellin also prevented the development of disease symptoms (e.g. exophthalmia). In some respects, preventing the development of disease symptoms is as important as preventing diseases-associated mortalities in that fish showing signs of disease

cannot be brought to market. In this respect severe financial losses may still entail even if livestock remain alive.

An apparent effect on fish upon administering r-flagellin vaccinates was a rapid increase in appetite/growth. A similar phenomenon has been observed for fish after administered probiotics (Brunt *et al*., 2007). Although vaccinates in this study were not comparatively larger when compared to controls, it should be noted that all fish were only monitored for a period of three weeks post-vaccination. As is the case with probiotics, a longer period of time (e.g. 6 to 8 weeks) is usually required before any obvious differences in size can be detectable. It would be interesting to repeat this experiment over a longer period of time as an increase in appetite would presumably increase growth rate and, as a result, increase turnover of livestock. Still, this is only speculative and requires further investigation.

4.9 Toxicity of native flagellin for rainbow trout

Applying r-flagellin via i.p. injection over a wide range of concentrations to rainbow trout (0 to 200 µg/fish) had no detrimental effects on health or behaviour. This again makes it an appealing vaccine in that livestock which may become accidentally overdosed with flagellin on a large scale by the aquaculturist would not result in mortalities. This aspect is crucial for the development of a new vaccine.

On the other hand, when native flagellin was administered to trout, relatively low protein concentrations ($\geq$ 100 µg/fish) caused mortalities. This was clearly independent of flagellin since very high levels of r-flagellin (above) did not have any detrimental effects on health. It could be speculated that contaminants, such as LPS or non-flagellin protein, were responsible for mortalities. Low MW contaminants which were visible in the native protein preparation (Fig 3.17) may be detrimental to fish health.

Nonetheless, another explanation may be put forward to explain why vaccination with native flagellin resulted in mortalities. As outlined in the results (Section 3.7), it was clear that fish did not recover from anaesthesia following sedation and i.p. injection. It should also be noted that all fish were sedated in anaesthetic at the same time before injecting groups with flagellin in an ascending order of concentrations. Thus it is likely that by the time higher concentrations (e.g. 100 µg/fish) were

administered, fish had already become overdosed in anaesthetic. This is a plausible explanation since all fish administered with r-flagellin at higher concentrations (e.g. 200 µg/fish), which involved a shorter sedation time in anaesthetic directly before injection, did not result in any mortalities. It should be stated that sedation of fish was still ensured before injection. Still, whatever the cause of death, the r-flagellin preparation is non-toxic for rainbow trout and could be used as a vaccine without detrimental effects on livestock.

4.10 Immunological aspect of protection associated with flagellin

The use of flagellin to protect against a flagellin-devoid pathogen is a paradoxical result. If flagellin is a target of the immune system in fish that have undergone vaccination with this protein, why should it then protect against a pathogen which does not produce this epitope? It may be that this very question or line-of-thought has prevented others from performing and/or publishing these results in the past. Although no immunological assays were performed in this study, there are nonetheless a number of features both within this study and that within published literature, which offers a rational explanation as to why flagellin protects against a non-motile pathogen.

Many vertebrates, including fish, possess both an innate and adaptive immune system. Unlike an adaptive immune response, the innate immune system is quick to react to an infection and is temperature independent (Ellis, 2001). Stimulation of an innate immune response is also not dependent on the recognition of specific molecules which are exclusively associated with any one pathogen. In this current study, protection against bacterial challenge was studied after 14 and 28 days post-vaccination with r-flagellin. This time period is clearly insufficient for an adaptive immune response insofar as antibody production against *Y. ruckeri* in rainbow trout does not occur until after 8 to 12 weeks post-vaccination (Deshmukh *et al.*, 2012). Furthermore, had flagellin induced an antibody response within this time period, it would not account for the levels of protection observed against a flagellin-devoid EX5 strain. Results therefore allude to an innate immune response, as protection is obtainable after a short period of time and is non-specific (i.e. not dependent upon flagellin expression by the invading pathogen). As noted by Ellis (2001), the innate immune system can be either constitutive (i.e. always present) or inducible

following vaccination/bacterial infection. Thus, flagellin is capable of inducing a protective immune response which can prevent the establishment of disease.

Whereas results thus far have demonstrated that flagellin is capable of inducing a protective immune response, it does not provide any detail on what specific aspects of the innate immune system are involved in providing this level of protection. However, there has been an extensive amount of research exerted in an attempt to understand the fish immune system, particularly regarding the response of the innate immune system to flagellin.

The innate immune system makes use of so-called pathogen recognition receptors (PRRs) or pathogen recognition proteins (PRPs) to detect the presence of conserved molecules generally associated with bacteria or viruses. Collectively these molecules are termed as pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular-patterns (MAMPs) (Palti, 2011). The recognition of PAMPs by PRRs ultimately results in the production of immune effector molecules (e.g. cytokines, chemokines and interferons) to inhibit and/or eliminate the invading pathogen. Flagellin is an example of such a PAMP and is widely recognised by PRRs in mammals, flies and plants as well as fish.

In humans, flagellin is recognised by Toll-like receptor 5 (TLR5), a PRR which is a member of the Toll-like receptor (TLR) family. This receptor is comprised of an extracellular N-terminus with a leucine-rich repeat region (LRR), a transmembrane (TM) domain and an intracellular C-terminus with a Toll/IL-1 receptor domain (TIR) (Palti *et al.*, 2011). Rainbow trout also carry TLR5 orthologues within the genome. However, unlike higher vertebrates (e.g. humans), rainbow trout genomes carry both a membrane-bound form of TLR5 (TLR5M) and a soluble form of the protein (TLR5S) (Tsujita *et al.*, 2004). Whereas TLR5M has all the previously described domains (e.g. LRR, TM and TIR), TLR5S isoforms contain only a LRR domain (Fig 4.1). Nonetheless, both rainbow trout TLR5 isoforms bind to and recognise flagellin (Tsujita et al., 2004, Hwang et al., 2010, Basu et al., 2012, Moon et al., 2011). Additionally, recognition of flagellin by TLR5 can have a positive feedback effect on the expression of soluble TLR5 (TLR5S), but less of an effect on the membrane form of the receptor (TLR5M) (Moon *et al.*, 2011, Tsujita *et al.*, 2004, Hwang *et al.*, 2010).

The recognition of flagellin in fish (Fig 4.1) can also result in the activation of downstream components including MyD88 (myeloid differentiation primary response protein), IRAK (interleukin-1 receptorassociated kinase), TRAF6 and nucleation factor NF-κB (Tsujita *et al*., 2004, Hwang *et al*., 2010, Basu *et al*., 2012, Hynes *et al*., 2011). This, in turn, results in the expression of pro-inflammatory cyotokines such as tumour necrosis factor-α (TNFα) and interleukin 1-β (IL-1β). Hynes *et al.* (2011) also found that expression of TNFα was significantly increased at days 2 and 4 post-vaccination. As noted by the authors, the production of TNFα following flagellin recognition is significant in that it is an important macrophage-activation factor which can stimulate nitric-oxide production, phagocytosis and respiratory burst activity in rainbow trout macrophages (Hardie *et al*., 1994). TNFα production can also increase chemotaxis of anterior kidney leucocytes and positively regulate the expression of IL-1β and COX2.

Published literature to date clearly demonstrates that flagellin can initiate a robust immune response towards bacterial infection. This is reinforced by work in this study as flagellin can prevent the development of ERM when used as a vaccine. In this respect, it is likely that administering flagellin prior to bacterial challenge can stimulate a robust innate immune response which essentially "primes" the host against bacterial infection. It is also possible that *Y. ruckeri* cells (serotype O1 or EX5) grown *in vitro* are not fully expressing all genes required for virulence and/or growth *in vivo*. Thus, when cultures are used as in the challenge model, bacteria are quickly and efficiently cleared by the immune system without adequate time to mount bacterial defences and/or acclimatise to the new environment. This would explain why the majority of fish (98%) vaccinated with r-flagellin did not show any symptoms associated with disease after challenging with the EX5 (R1) strain. It is therefore possible that protection in a commercial setting, when the challenge comes from bacteria derived from fish, may be not as robust as that seen here. Nevertheless, it does show that the innate immune system is a powerful aspect of rainbow trout immunity which in itself can protect against ERM.

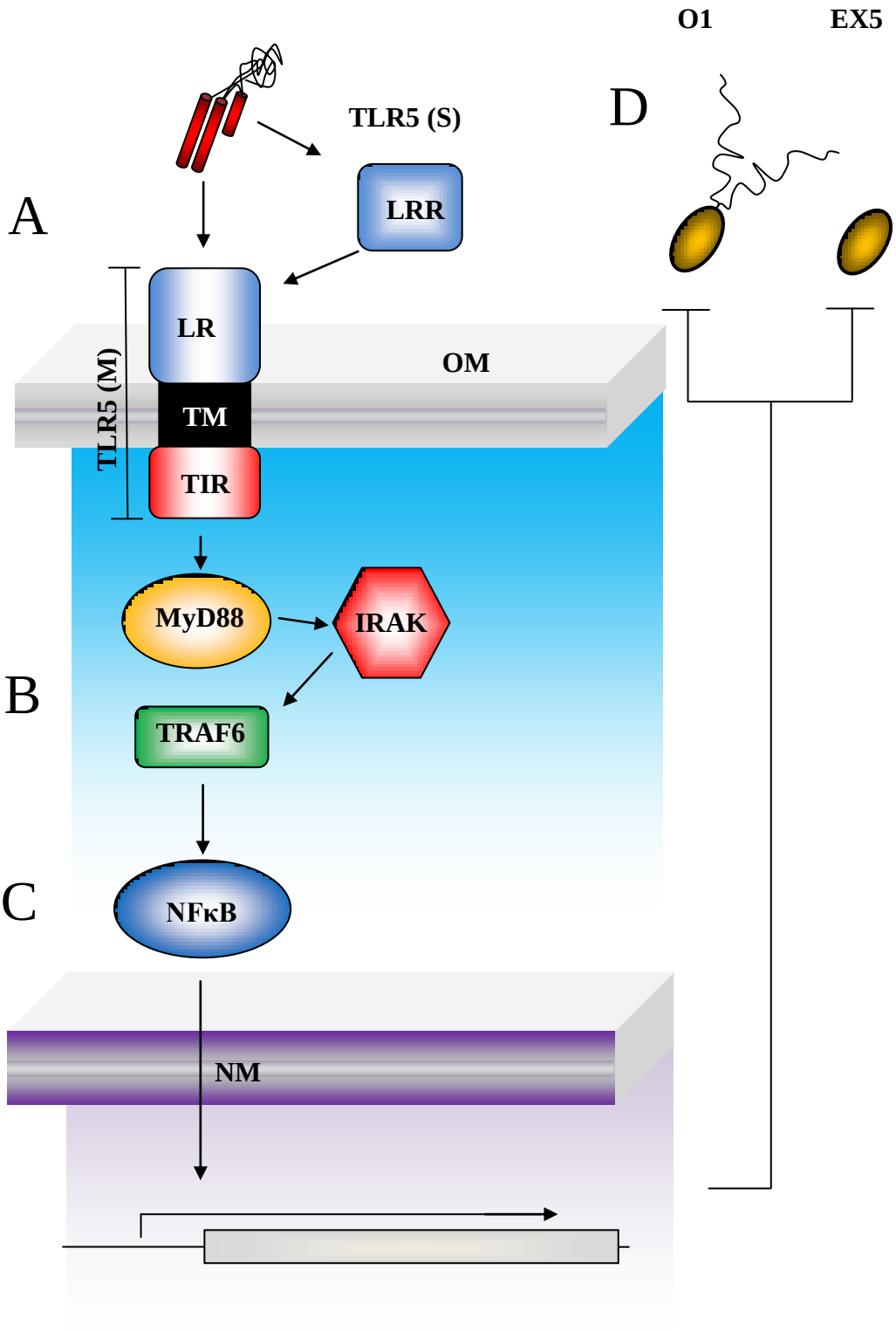


Figure 4.1: Recognition of flagellin by TLR5 in rainbow trout and potential downstream signalling pathways leading to protection against challenge with a virulent serotype O1 or EX5 *Y. ruckeri* strain. Flagellin is recognised by membrane (TLR5M) or soluble (TLR5S) TLR5 (**A**) which initiates the downstream activation of the MyD88 dependent pathway (**B**). This ultimately results in the activation of NFκB (**C**) and its localisation to the nucleus whereby it initiates the expression of

pro-inflammatory cytokines (e.g. IL-1β and TNFα). This would result in a pro-inflammatory response which would lead to the repression and/or clearing of *Y. ruckeri* (serotype O1 and EX5).

4.11 Flagellin as a vaccine in other research

Given the increasing amount of information regarding the immunological response to flagellin in fish, other researchers have endeavoured to investigate the potential role of this protein as a vaccine. However, unlike results outlined in this work, all published research to date has involved administering flagellin with an adjuvant (e.g. Freunds complete adjuvant) in an attempt to stimulate antibody production against challenge with a flagellin-producing strain. For example, recombinant flagellin from *Piscirickettsia salmonis* (Wilhelm et al., 2006), *V. alginolyticus* (Liang et al., 2010) and *A. hydrophila* (Yeh and Klesius, 2011) have been shown to induce antibody production when injected to fish with adjuvant. Any protection conferred against challenge to motile (= flagellin-producing) strains was again linked with an increase in antibody titres. In light of current research it is entirely possible that flagellin, in addition to initiating an adaptive immune response, is also eliciting a non-specific immune response in fish.

As well using the flagellin protein in a vaccine formulation, workers have also used DNA vaccines harbouring genes encoding flagellin. This technique was used with some success by Liang *et al.* (2011) to specifically protect red snapper (*Lutjanus sanguineus*) against *V. alginolyticus* infection, again with the ultimate goal of stimulating antibody production. In contrast, Jiao *et al.* (2009) suggested that a DNA vaccine containing the flagellin gene (*fliC*), expressed within Japanese flounder, did not adequately protect against *E. tarda*. These researchers also found that administering recombinant flagellin protein did not sufficiently protect (70% mortality) against challenge. This is in contrast with results outlined in this thesis whereby recombinant flagellin protected fish against a motile bacterium. However, work by Jiao *et al.* (2009) involved a different flagellin, fish and bacterial species. It also made use of a *Bacillus* spp. B187 adjuvant in the flagellin preparation.

Studies exploring the use of flagellin as a component of a vaccine have similarly been performed on terrestrial animals such as rats (Shi *et al.*, 2012), mice (Hong *et al.*, 2012), chickens (Chaung *et al.*, 2011) and monkeys (Weimer *et al.*, 2009).

However, these experiments have all involved the use of flagellin as an adjuvant in an attempt to stimulate a pro-inflammatory response which would precede antibody production against viral or bacterial antigens. In this respect, flagellin research regarding its use as a vaccine in mammals is not dissimilar to that in fish since researchers are ultimately trying to initiate a humoral response towards an infective agent. A notable study by Lee *et al*. (2006) showed that flagellin (FlaB) from the bacterial fish pathogen *V. vulnificus* has potent mucosal adjuvant activities when intranasally administered to mice. What is more, administering FlaB in conjunction with a tetanus toxoid (TT) resulted in the production of specific anti-TT immunoglobulin A (IgA). Lee *et al*. (2006) also demonstrated that flagellin was bound to TLR5 which resulted in the activation of TNF-κB and IL-8. Thus while flagellin, like in fish, has the potential to elicit a strong immune response, no research has been performed concerning flagellin as a standalone vaccine.

4.12 Non-specific vaccines for fish

In aquaculture, so-called non-specific immunostimulants or immunomodulators have gained an increasing amount of attention in recent years. While flagellin has consistently been referred to as a vaccine for the purposes of this work, it would also match the criteria of an immunostimulant. These can take a number of forms which broadly include probiotics and dietary supplements that apparently result in the stimulation of the innate immune system. How these compounds or bacteria result in a protective immune response is a matter open to some debate. Probiotics, which have been shown to be effective against ERM (Section 1.16.2), are broadly considered to be live bacteria that, when administered to fish, has a beneficial effect on health and in combatting against disease. This terminology may not strictly hold true, however, since Sharifuzzaman *et al*. (2011) showed that administering crude cell wall proteins (200 to 250 µg/fish) from probiotic bacteria could offer protection against challenge with *V. anguillarum* (RPS = 80 to 87%). It is possible that immunostimulatory molecules or MAMPs produced by these strains are stimulating an immune response which is not dissimilar to that induced by flagellin administration. More recently Li *et al*. (2012) showed that vaccinating zebrafish with glyceraldehyde-3-phosphate dehydrogenase conferred some level of cross-protection against a range of fish pathogens including *A. hydrophila*, *V. anguillarum* and *E. tarda*. However, levels of cross protection was relatively low (RPS = ~60%). Taking RPS values obtained in this study using flagellin into consideration, it would

reinforce the need to test the ability of this protein to protect against the range of pathogens outlined by Li *et al*. (2012). It is noteworthy that while probiotics are known to stimulate an innate immune response, the role of food supplements in stimulating such a response is well understood. There is much debate over the role of improved nutrition in combatting disease rather than the compound directly stimulating an immune response.

4.13 Flagellin as a commercial, non-specific vaccine

Results outlined in this work are promising regarding the use of flagellin as a non-specific vaccine for protecting against disease in fish. However, there are a number of areas not approached in this work which would have to be addressed before flagellin could be used as a vaccine on a commercial basis.

4.13.1 Pathogen specificity and length of protection

Work outlined here is limited in that only two specific types of *Y. ruckeri* strains were used in the challenge model after 14 and 28 days post-vaccination. As previously discussed, protection conferred by flagellin towards bacteria was non-specific. It would be of interest to investigate the protective properties of this protein against a range of motile and non-motile bacterial pathogens of fish which are either Gram-negative (e.g. *V. anguillarum* and *A. hydrophila*) or Gram-positive (e.g. *Edwardsiella tarda* and *Streptococcus iniae*). Exploring the protective effects, if any, of this vaccine against viral infection should also be considered as the number of vaccines currently available against viral agents are limited (Austin *et al.*, 2007). Actually, should a viral outbreak occur in livestock, there is little option but to cull infected livestock as many compounds used to treat bacterial infections (e.g. antibiotics) are completely ineffective towards curtailing viruses.

Length of protection conferred by this vaccine should also be investigated as an innate immune response may subside after some time. For example, Deschmukh *et al*. (2012) has shown that levels of protection 8 or 12 weeks post-vaccination can result in a significant drop in the levels of protection towards challenge. Should vaccination induce an innate immune response, it is then possible that levels of protection against challenge 8 or 12 weeks would subside. A similar result might be observed when using flagellin as a vaccine, thereby necessitating long term

vaccination studies (e.g. challenge after 8 or 12 weeks post-vaccination with flagellin).

In addition to preventing the development of disease, it would be interesting to investigate the immunostimulatory properties of flagellin on fish showing signs of disease. For example, it is widely known that ERM is associated with stressed or overpopulated livestock, thus leading to the immunocompromised state. Thus reducing the size of stocks and applying flagellin to fish showing early signs of disease may stimulate a protective immune response and reduce mortality rates. In this way, excessive loss of livestock may be prevented without significant financial losses for the aquaculturist. However, this is again purely speculative.

4.13.2 Vaccine application

Injection is an effective method for determining the protective properties of a vaccine, although it is costly, time consuming and impractical for large-scale immunizations. As stated earlier, administering vaccines by injection to large and/or valuable fish is still a plausible option. However, other methods of vaccine application would have to be considered if r-flagellin was to be administered on a large scale. As noted in a recent review by Plant and LaPatra (2011), coating or mixing feed with antigen can have the added advantage of being stress free for livestock and easier for applying to large numbers of fish. This method has a number of problems such as determining the protective dosage and ensuring that each fish receives an adequate amount of antigen can be problematic. Antigen may also be broken down by digestive enzymes in the lumen of the gut and consequently not have a demonstrable effect on the fish immune system (Plant and LaPatra, 2011).

Whereas small, immunogenic peptides could probably withstand host digestive enzymes, it is unlikely that each fish would consume a similar amount of food. The only logical method of ensuring that each trout obtains enough of the recombinant antigen would be to significantly increase the amount added to feed. This poses a number of difficulties. Firstly, a large amount of recombinant protein or peptides would be required, thus increasing the overall cost of vaccine production. Secondly, large amounts of this product in feed may make it unpalatable for fish.

Taking into consideration all the methods outlined by Plant and LaPatra (2011), it is likely that vaccination via immersion would be the method of choice for applying the recombinant *Y. ruckeri* flagellin protein *en masse*. This form of vaccine delivery, which is shown diagrammatically in the appendix (Fig 5.9), has a number of features which make it superior over other methods. Unlike injection, immersion would limit the amount of stress imposed on livestock, is faster, and would not require personnel trained in fish injection. There is also the added advantage of being able to estimate the dose of antigen required for conferring protection. For example, the ability to reduce the volume of water to a certain level for a given number of fish would ensure each trout is sufficiently immunized. This method is currently used in vaccinating against a number of diseases, including ERM, and thus flagellin for convenience could be administered at the same time without further costs for the aquaculturist. In principle, the protein could be added directly to the vaccination tank without having to mix with feed. Furthermore, as the vaccine is proteinaceous in nature, it would be stable for a number of years either by freezing, lyophilisation or in liquid form following the addition of a preservative (e.g. sodium azide). Large amounts of vaccine could be produced at any one given time and stored indefinitely until required.

In this study, as for vaccination, all challenges were performed by i.p. injection. Even though this method is again adequate for evaluating the protective properties of a given vaccine, it does not mimic natural infection. Injection also essentially bypasses the first line of defence (i.e. skin, antimicrobial peptides, and digestive enzymes) which presumably plays a major role in preventing infection. Thus, in addition to investigating administering flagellin via immersion, fish should be challenged by immersion.

4.13.3 Vaccine structure

As indicated in the results, it is likely that the conserved N- and/or C- terminal is contributing to protection. Some possible structures which could be considered are shown in the appendix (Fig 5.10). Constructs could be created using different primer sets to specifically overexpress immunostimulatory peptides which may reduce the amount of protein required to confer a protective immune response. Smith *et al*. (2003) has shown that a 13 amino-acid residue, which is conserved between different flagellin proteins and required for motility and proto-filament formation,

can sufficiently stimulate TLR5 recognition even at very low concentrations e.g. 10 ng/ml. With further modifications it would seem that protection and/or immune stimulation could probably be achieved after modifying the vector construct. Thus there are potentially some exciting prospects regarding flagellin as a vaccine/immunostimulant.

4.14 Vaccine failure and EX5 outbreaks

Although flagellin is effective in preventing ERM when used as a sub-unit vaccine, and whereas the non-motile phenotype has probably not arisen due to a selection pressure on flagellin, it does not explain as to why EX5 isolates originally caused disease in rainbow trout previously vaccinated against this disease. A possible explanation using data generated in this work, while still not conclusive evidence, may be put forward to explain why this strain of *Y. ruckeri* caused disease.

Like Austin *et al.* (2003), this study confirmed that vaccinating fish with a serotype O1 strain can offer some levels of protection 28 days post-vaccination against challenge with a serotype O1 *Y. ruckeri* isolate. However, it was shown that fish vaccinated with a serotype O1 strain were susceptible to infection with EX5 cells. In this respect, data from these vaccine/challenge experiments represent the so-called "vaccine failure" of the monovalent (= serotype O1) strain in adequately protecting against EX5 infection first described by Austin *et al.* (2003). Moreover, both work in this study and that by Austin *et al.* (2003) demonstrated that vaccination with an EX5 strain did not confer high levels of protection against challenge with an EX5 strain.

On the other hand, in contrast to Austin *et al*. (2003), vaccination studies using the EX5 strain as a vaccine showed that trout were sufficiently protected against challenge with a serotype O1 (YR1) *Y. ruckeri* strain (RPS = 95%). This shows that inactivated EX5 cells can effectively stimulate a protective immune response against a serotype O1 isolate. However, EX5 themselves have a greater tolerance towards such a response, suggesting that they are able to withstand the effects of the immune system and persist within the host.

4.14.1 Link between virulence and vaccine-induced protection

The serotype O1 *Y. ruckeri* isolate (BA19), which was used in this study to create a flagellin devoid strain (BA19/Tn-RL27), is the strain used in the original (= monovalent) ERM vaccine which consequently failed to protect against EX5 isolates (Professor. B. Austin, University of Stirling, *personal communication*). An attempt to obtain a fresh culture from the vaccine manufacturers was unsuccessful, although the BA19 strain was available within as a lyophilised culture from 1983 and remained viable when grown *in vitro*. This strain was originally obtained from rainbow trout displaying signs of ERM in the UK during the 1960s and has presumably been used in the vaccine formulation until the recent outbreak of EX5 strains in vaccinated livestock. It should also be noted that this strain originally conferred excellent levels of protection (RPS $\geq$ 95%) against challenge with the serotype O1 strain (Section 1.17.1).

Interestingly, work in this study using the serotype O1 (BA19) isolate showed it to be completely avirulent for rainbow trout since challenging naïve fish with this isolate (i.e. 1 x 10^5, 10^6, 10^7, 10^8, 10^9 cells/ml; N = 5 x 5) did not induce any mortalities 2 weeks post-challenge (100% survival). Evidently the BA19 strain has moved from being virulent to avirulent since it was previously sourced from diseased fish (above). Why this *Y. ruckeri* strain has lost virulence remains unknown, however, routine sub-culturing *in vitro* is known to have a detrimental effect on the pathogenicity of this organism. For example, fresh *Y. ruckeri* EX5 isolates sourced directly from diseased rainbow trout were found to be highly virulent towards naïve fish as administering 1 x 10^5 EX5 cells/fish resulted in 100% mortalities after 4 days post-challenge (Austin *et al*., 2003). In fact as few as 1 x 10^3 EX5 cells/fish could cause mortalities (Dr D. Austin, Heriot-Watt University, *personal communication*). However, even after passing through fish, EX5 isolates were reduced in virulence towards rainbow trout as 4 x 10^5 cells/fish resulted in only 52% mortalities after 2 weeks post-challenge (this study).

It could be hypothesised that highly virulent/aggressive *Y. ruckeri* isolates sourced directly from naturally infected fish are adapted for growth and persistence *in vivo*. For example, it is now widely accepted that one of the selective pressures or regulators of virulence *in vivo* is iron and temperature limitation (Tobback *et al*., 2007). As the majority of iron is bound within host cells as protein-iron complexes

(e.g. haemoproteins), pathogenic bacteria must produce virulence factors such as siderophores, haemolysins, proteases and iron-regulated outer membrane proteins (IROMPs) in order to obtain this essential nutrient (Tobback *et al*., 2007). Research to date has shown that when *Y. ruckeri* is cultured *in vitro* in nutrient rich media (e.g. TSB or NB) at the optimum temperature of growth (i.e. 28ºC), production of these molecules is significantly repressed, sometimes even to undetectable levels (Tobback *et al*., 2007). An important observation in this study, although not directly quantified, found that EX5 strains grew better at very low temperatures (i.e. 4ºC) compared to the serotype O1 (BA19) strain. Moreover, the EX5 strain was far more efficient in growing under iron and/or temperature limiting (16ºC) condition compared to the serotype O1 (BA19) isolate. Nonetheless, the serotype O1 (BA19) could be "trained" to grow under virulence-inducing conditions, although a potential increase in pathogenicity for fish was not investigated. It is conceivable that continuously culturing/sub-culturing serotype O1 (BA19) bacteria on nutrient rich media has effectively removed any selection pressure that lends towards a virulent phenotype. In some cases it is possible to regain or increase virulence of *Y. ruckeri* strains via multiple passages through fish. It is worth noting, however, that while iron and temperature are considered to be influencing factors of virulence for *Y. ruckeri in vivo*, it is highly probable that other unknown environmental cues are influencing virulence gene expression. This is considered by many authors to be the ultimate downfall in *in vitro* experimentation for this fish pathogen (Tobback *et al*., 2007).

Understanding why certain fish vaccines confer better levels of protection against different forms of bacterial pathogens is often a contentious area of debate, principally because the roles of the innate and/or humoral immune system have not been definitively clarified within the literature. However, it is now becoming apparent from recent publications that vaccination with inactivated bacteria (or attenuated live forms of the pathogen) can result in the upregulation of genes (e.g. pro-inflammatory cytokines) which are associated with innate immunity (Section 1.18.1). It is likely that protection observed in this study is associated with this aspect of the immune system since challenge was performed 28 days post-vaccination, a time period which is insufficient for antibody production in rainbow trout (Raida *et al*., 2011b). Furthermore, the use of flagellin in this work as a

component of a sub-unit vaccine confirms the involvement of the innate immune system in protection towards bacterial challenge.

Assuming that the innate immune system is stimulated upon vaccination, it suggests that PAMPs produced by *Y. ruckeri*, which are present within the inactivated whole-cell vaccine preparation, are involved in conferring protection. In this respect, PAMPs required for virulence are in themselves recognised by the innate immune system. Thus the avirulent serotype O1 (BA19) strain, which is possibly not expressing the same amount and/or combination of PAMPs required to stimulate a robust immune response, does not result in significant levels of protection against bacterial challenge. A similar situation has been observed for *Helicobacter pylori* whereby routine sub-culturing on solid media has resulted in the loss of antigenic O side-chain expression. If work by Tinsley *et al*. (2011b) is correct, then it is possible that serotype O1 (BA19) isolates are not producing a component of LPS which is required for stimulating an innate immune response. Conversely, highly virulent EX5 isolates could be expressing these immunostimulatory molecules which are, in themselves, required for virulence. It could be theorised that when inactivated EX5 cells are used as a vaccine, PAMPs produced by these strains result in the stimulation of the innate immune system which can subsequently protect against bacterial challenge. This explanation would fit results outlined in this study, although published research has yet to prove that this is in fact the case.

As discussed earlier, EX5 *Y. ruckeri* cells are able to resist the immune responses of fish vaccinated with the monovalent vaccine. Even fish vaccinated with the EX5 strain are still, to some extent, susceptible to infection. This form of resistance to an immune response is indicative of serum resistance; an aspect of virulence which is associated with *Y. ruckeri* pathogenicity (Section 1.9.1). It has been suggested that serum resistance is an essential requirement for virulence by pathogenic serotype O1 strains *in vivo* (Section 1.9.1). Interestingly, although this aspect of virulence is not fully understood, it is possible that serum resistance is associated with LPS. It could be theorised that extensive vaccination has driven the development of highly aggressive, serum resistant EX5 isolates.

4.15 EX5 and OmpA overexpression

Upon fractionating WCPs by SDS-PAGE, it was strikingly clear that a 30 to 40 kDa protein was overexpressed by EX5 isolates. Although this protein was not unique to this *Y. ruckeri* strain (different serotypes were also expressing this protein), they did not reach the levels of overexpression observed by EX5 strains. This result was also observed by Wheeler *et al*. (2009), who separated crude OMPs by SDS-PAGE, although the identity of the protein was not established. Of note, Davies *et al*. (1990) also described a strain with an overexpressed protein of 30 to 40 kDa, although motility and/or virulence for this strain particular isolate was not stated.

In order to identify this protein, WCPs were purified and separated by 2D SDS-PAGE. It was evidently clear that four major protein spots within the region of 30 to 40 kDa were overexpressed by EX5 strains when compared to other serotype O1 isolates. Analysis of these protein spots following trypsin digests/Maldi-ToF mass spectrometry indicated that they were similar to outer membrane protein A (OmpA) of *Y. enterocolitica*.

The overexpression of OmpA was interesting insofar as this protein has been shown to have a role in virulence for a number of pathogens belonging to the Enterobacteriaceae (Krishnan and Prasadarao, 2012). For example, clinical isolates of pathogenic *E. coli* K1 require OmpA to resist against serum killing and aid in the attachment/invasion of host tissues (Krishnan and Prasadarao, 2012). It could be postulated that OmpA may compensate for the lack of flagellin production in establishing attachment and/or invasion of fish tissues. EX5 is also able to cause disease in fish vaccinated with the monovalent vaccine, suggesting that it is capable of resisting an immune response. Indeed, it has been established that resistance to serum-mediated killing by *Y. ruckeri* is important for virulence (Section 1.9.1). It is conceivable that OmpA overexpression by EX5 might be contributing to serum resistance, particularly since all the molecules involved in this aspect of virulence are currently unknown. Furthermore, OmpA overexpression by EX5 may also aid in establishment and/or maintenance of biofilms as these structures are potentially a source of re-infection and persistence within the environment (Coquet *et al.*, 2002a, Coquet *et al.*, 2002b).

To ascertain what role OmpA might have in virulence for *Y. ruckeri* EX5 cells towards rainbow trout, an attempt was made to disrupt the gene encoding this outer membrane protein (*ompA*). Since the transconjugation system described by Metcalf *et al*. (2002) was optimised for creating *Y. ruckeri* transconjugants, and as it was successfully used to screen for a non-motile serotype O1 (BA19/Tn-RL27) mutant, an attempt was made to screen for a *Y. ruckeri* EX5 transconjugant carrying a transposon within the *ompA* gene (*ompA*::Tn-RL27). Unlike the previous screening process, which employed a specific phenotype to isolate a transconjugant, screening for a transposon within a specific gene initially posed a problem. However, both the transposon flanking/sequencing primers TPNRL17-1 and TPNRL13-2 were readily available, as were the *ompA* forward (OMPA-F) and reverse (OMPA-R) primers. Therefore, pooled transconjugants could theoretically be screened by PCR and detected using Southern blotting/hybridization with a DIG-labelled PCR probe. Unfortunately, out of 4800 transconjugants, none contained the Tn-RL27 transposon within the *ompA* gene.

One possible explanation as to why the transposon screening process failed to isolate a transconjugant of interest is that the *ompA* gene is not a region which is susceptible to insertion (i.e. "hot-spot"). The isolation of a non-motile *Y. ruckeri* strain carrying a transposon within the same site as that of the non-motile transconjugant isolated by Evenhuis *et al*. (2009) would reinforce this theory. Another explanation is that the OmpA protein is physiologically essential for maintaining bacterial stability, and upon its disruption results in the loss of viability. The latter explanation would seem unlikely, particularly since viable *E. coli* strains with mutations in the *ompA* gene can be readily obtained (Krishnan and Prasadarao, 2012). It is also possible that *Y. ruckeri* transconjugants which carry a transposon within the *ompA* gene are reduced in their ability to compete with other strains during the conjugation stage and therefore not obtainable following this process. It does suggest that this conjugation system is insufficient or unable to produce the mutant of interest. Future attempts at creating disruptions in the *Y. ruckeri ompA* gene should be carried out using site-directed mutagenesis in a bid to better understand the role of this protein in virulence towards fish.

4.16 Relationship between *Y. ruckeri* and other *Yersinia* spp.

The question as to the correct placing of *Yersinia ruckeri* within the genus *Yersinia* has long been debated. Discussions over the correct genus for this organism can be linked back to initial work carried out by Ewing *et al.* (1978). A broad study by Kotetishvili *et al.* (2005) using MLST showed that *Y. ruckeri* was genetically distant to all other *Yersinia* spp. and may even substantiate a new genus. However, DNA sequencing in this study has highlighted a close phylogenetic relationship between *Y. ruckeri* and *Y. enterocolitica*. For example, BLAST analysis of two previously unidentified *Y. ruckeri* ORFs (*fliC* and *ompA*) most closely resemble and shared a high level of identity ($\geq$85%) to homologues within the *Y. enterocolitica* genome (*flaA* and *ompA*). While it cannot be assumed outright that *Y. ruckeri* is genetically a *Yersinia* spp. based on two genes alone, it does indicate that there are some genetic similarities between *Y. ruckeri* and *Y. enterocolitica*. Work should again be taken up on definitively allocating this species within a new genus or maintaining it as a *Yersinia* spp.

4.17 Concluding remarks

Y. ruckeri is a successful pathogen of fish and one which will undoubtedly continue to cause disease in salmonids, particularly rainbow trout. While some consider the current bivalent vaccine to be superior over the monovalent vaccine, research into developing new and/or novel methods of control should not stop. As was the case with the monovalent vaccine, it is likely that the over application of the bivalent vaccine, coupled with the intensification of aquaculture, will drive the evolution of highly aggressive *Y. ruckeri* strains which can resist vaccination. Thus, novel vaccines will be required to prevent disease in the near future. Results from this study suggest that flagellin may be a potential vaccine candidate against ERM, although long-term research will have to be carried out before any attempts are made to commercialise this vaccine. Nonetheless, current data are promising, and research in the near future regarding this fish pathogen will undoubtedly be exciting in fields of both microbiology and immunology.

4.18 Conclusions

1. *Y. ruckeri* EX5 isolates are non-motile and do not produce detectable flagellin under any of the standard conditions used to cultivate the pathogen.

2. The genome of the EX5 strain carries the gene encoding flagellin (*fliC*), indicating that the organism was once motile.

3. Flagellin is not the main immunostimulatory molecule in the monovalent vaccine. It would seem unlikely that extensive vaccination against a motile (i.e. flagellin-producing) strain exerted a selection pressure for a non-motile phenotype.

4. *Y. ruckeri* flagellin is an effective sub-unit vaccine against a flagellin-producing serotype O1 and flagellin-devoid EX5 *Y. ruckeri* strain.

5. The ORF encoding *Y. ruckeri* flagellin (*fliC*) was successfully cloned, and shown to be 1,284 bp long. Flagellin from this species was predicted to encode a 46.77 kDa protein (452 amino acids) which shares a high level of identity (82%) with flagellin from *Y. enterocolitica*.

6. Recombinant flagellin (r-flagellin) could be over-expressed to give relatively high yields (40 mg/L) and purified as insoluble inclusion bodies or soluble flagellin when passed through an IMAC column.

7. Recombinant r-flagellin was safe for use in fish at very high concentrations (200 µg/fish) without any detrimental effects on fish health. Evidence suggests that this protein may actually stimulate health and increase appetite.

8. Virulent EX5 *Y. ruckeri* strains are associated with the overexpression of outer membrane protein OmpA.

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APPENDIX

Table 5.1: Composition of media used to cultivate bacterial strains. All media were autoclaved (121°C; 15 min) and cooled (~ 50°C) before use.

Media	*Composition (grams per Litre)*	
Liquid media:		
Columbia broth	Biopeptone	10.0 g
	Dextrose	2.5 g
	Ferrous sulphate	0.02g
	Heart infusion powder	3.0 g
	L-Cysteine hydrochloride	0.1 g
	Magnesium sulphate	0.1 g
	NaCl	5.0 g
	Peptone	10.0 g
	Sodium carbonate	0.6 g
	Tris (Hydroxymethyl) aminomethane	0.83 g
	Tris (Hydroxymethyl) aminomethane-HCl	2.86 g
	Final pH: 7.3	
Luria-Bertani broth (LB)	NaCl	10.0 g
	Tryptone	10.0 g
	Yeast extract	5.0 g
	Final pH: 7.2	
Nutrient broth (NB)	D-glucose	1.0 g
	NaCl	6.0 g
	Peptone	15.0 g
	Yeast extract	3.0 g
	Final pH: 7.5	
Tryptone soya broth (TSB)	Dextrose	2.5 g
	Dipotassium phosphate	2.5 g
	Enzymatic digest of casein	17.0 g
	Enzymatic digest of soybean meal	3.0 g
	NaCl	5.0 g
	Final pH: 7.3	
Solid media:		
Luria-Bertani agar (LA)	LB	25.0 g
	Bacteriological agar No 1	10.0 g
	Final pH: 7.2	

Nutrient agar (NA)	NB	25.0 g
	Bacteriological agar No 1	10.0 g
	Final pH: 7.5	
Tryptone soya agar (TSA)	TSB	30.0 g
	Bacteriological agar No 1	10.0 g
	Final pH: 7.3	

Table 5.2: Susceptibility of selected bacterial isolates towards a range of antibiotics.

	Bacterial strains			
	Y. ruckeri		***E. coli***	
Antibiotic	***BA19***	***R1***	***BW2020767/pRL27***	***S17λ pir/pLM1***
M13 Ring:				
Chloramphenicol (25 µg)	S	S	S	S
Erythromycin (5 µg)	R	R	R	R
Fusidic acid (10 µg)	R	R	R	R
Oxacillin (5 µg)	R	R	R	R
Novobiocin (5 µg)	R	R	R	R
Penicillin G (1 unit)	R	R	R	R
Streptomycin (10 µg)	S	S	I	R
Tetracycline (25 µg)	S	S	S	S
M26 Ring:				
Ampicillin (25 µg)	S	S	S	S
Chloramphenicol (50 µg)	S	S	S	S
Colistin sulphate (100 µg)	S	S	S	S
Kanamycin (30 µg)	S	S	R	S
Nalidixic acid (30 µg)	S	S	S	S
Nitrofurantoin (50 µg)	I	I	S	S
Streptomycin (25 µg)	S	S	I	R
Tetracycline (100 µg)	S	S	S	S
Gentamicin (50 µg)	S	S	S	R

Key: S = Sensitive; I = Intermediate sensitivity; R = Resistant.

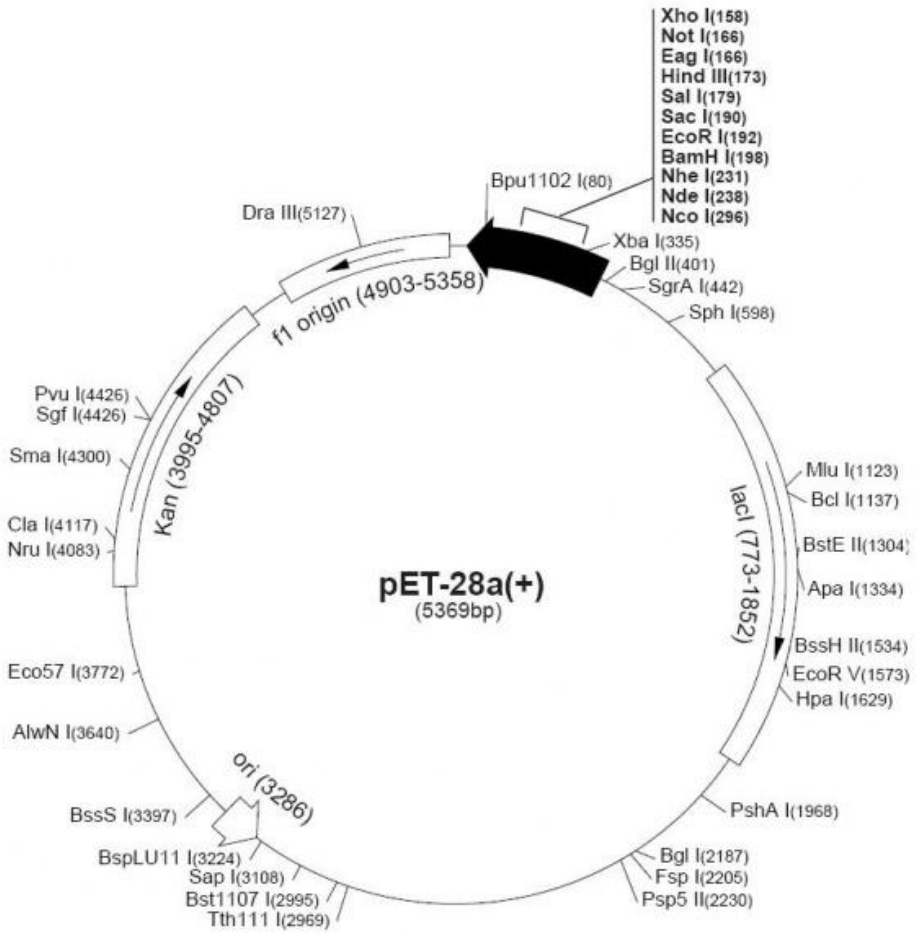

Figure 5.1: Restriction plasmid map of the pET-38-b vector (Novagen) used in this study to clone the *Y. ruckeri* flagellin gene (*fliC*).

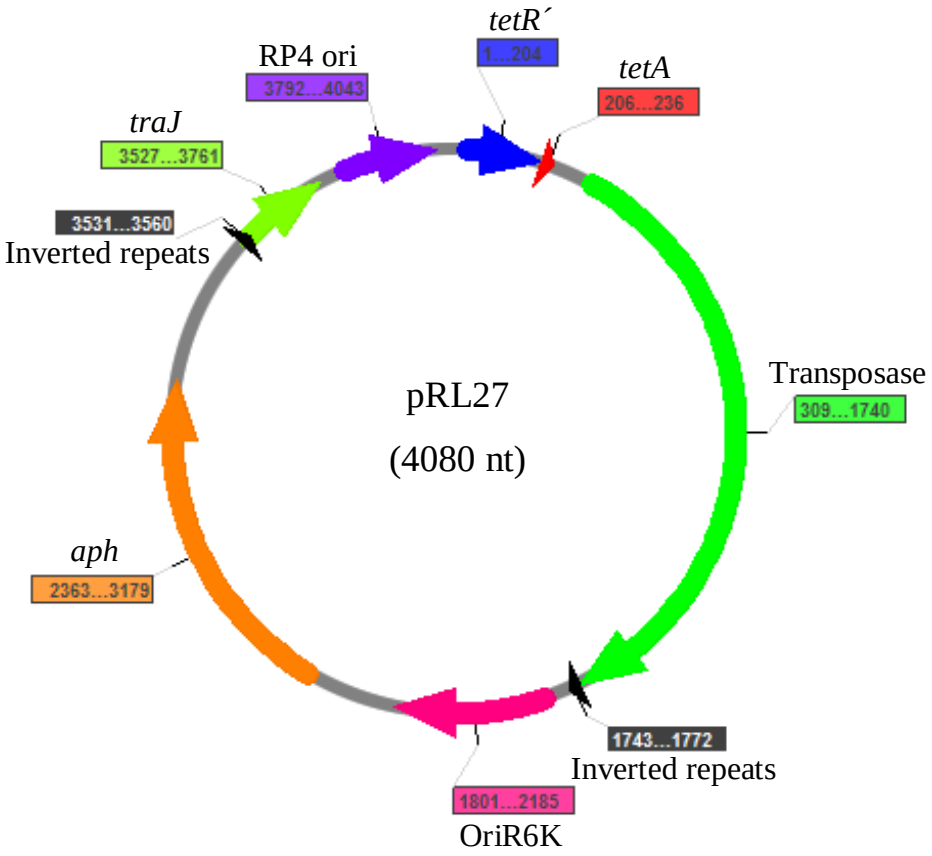

Figure 5.2: Plasmid map of the pRL27 vector used in this study to create *Y. ruckeri* transconjugants. Graphics were designed using SerialCloner (V2.1).

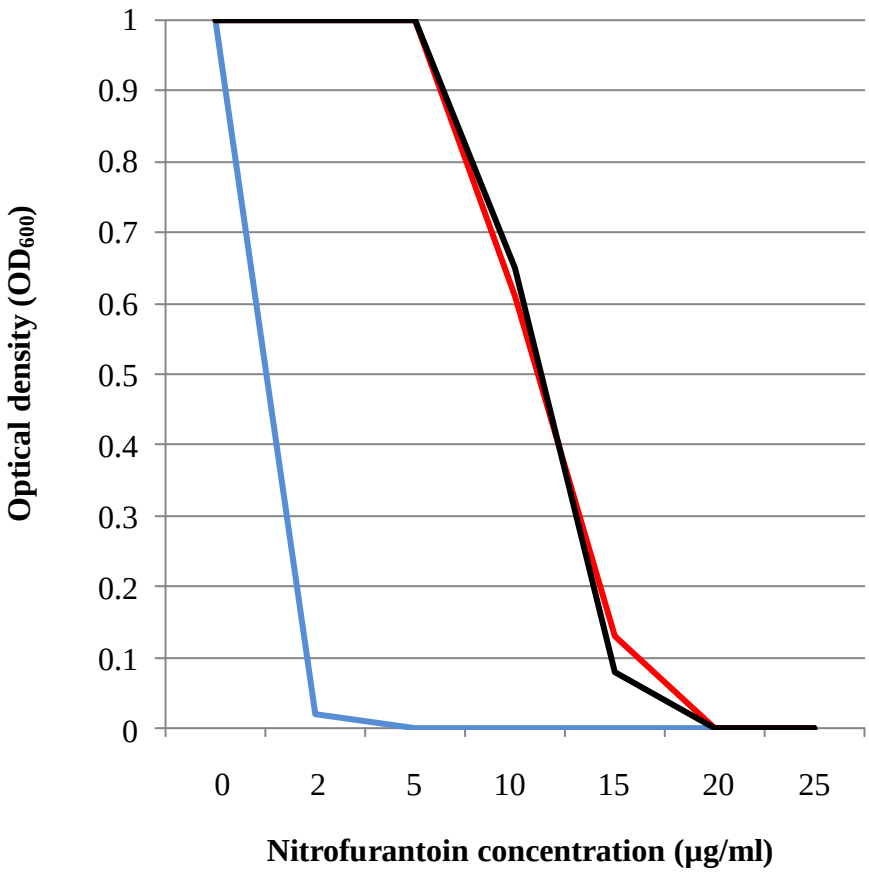

Figure 5.3: Determination of minimum inhibitory concentrations (MICs) for bacterial isolates towards nitrofurantoin. Optical density values for each concentration of nitrofurantion used in this work for the *E. coli* donor (BW2020767/pRL27) (■) and *Y. ruckeri* host strains serotype O1 (BA19) (■) and EX5 (R1) (■) is shown. Data represented above are from the mean of five different growth experiments.

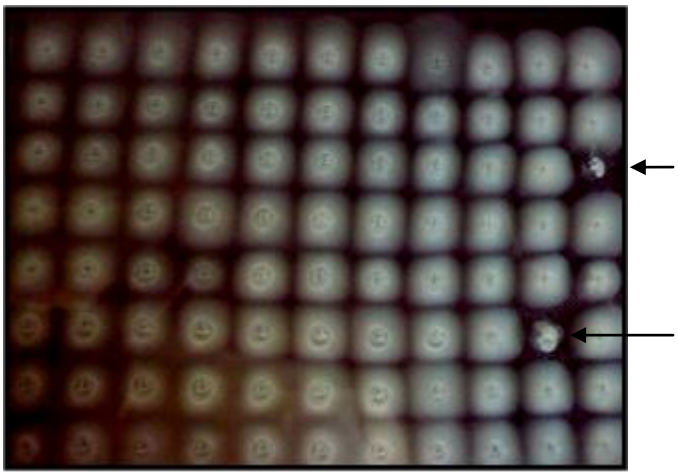

Figure 5.4: Large scale screening for non-motile serotype O1 (BA19) *Y. ruckeri* transconjugants. Cultures were stab inoculated from liquid cultures onto motility media (Columbia broth + 0.3% [w/v] agar) using a multi-well replicator. Two potentially non-motile mutants are clearly visible (indicated).

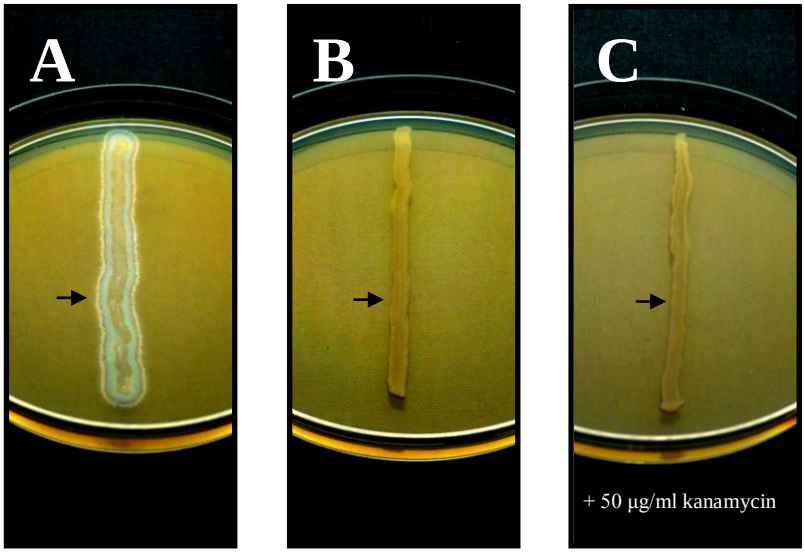

Figure 5.5: Lipase production and secretion by *Y. ruckeri* strains on TSA plates supplemented with 1% (v/v) Tween 20. Single colonies were streaked onto these plates before incubating at 28°C for 48 h. Lipase production by the motile serotype O1 (BA19) *Y. ruckeri* strain is visible (**A**), whereas the phenotype is not observable for both the EX5 isolate (**B**) and non-motile serotype O1 (BA19/Tn-RL27) transconjugant (**C**).

ATTTTAAAGACACGCAGTGGCAGGTACTTGCCGGTCCGATCCTGATATTAATGATTCTGTCGATGATG
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ATTGCTGGTCGCGATGTTTACCAAACGCACGCTGGAATTTGCTGCATTCCCGACCATTTTGTTGTTTT
CCACCTTATTACGTCTATCctgtctcttatacacatctcaaccATCATCGATGAATTCGAGCTCGGTA
CCCCCCC*ATGTCAGCCGTTAAGTGTTCCTGTGTCACTCAAAATTGCTTTGAGAGGCTCTAAGGGCTTC*
TCAGTGCGTTACATCCCTGGCTTGTTGTCCACAACCGTTAAACCTTAAAAGCTTTAAAAGCCTTATAT
ATTCTTTTTTTTCTTATAAAACTTAAAACCTTAGAGGCTATTTAAGTTGCTGATTTATATTAATTTTA
*TTGTTCAAACATGAGAGCTTAGTACGTGAAACATGAGAGCTTAGTACGTTAGCCA*TGAGAGCTTAGTA
CGTTAGCCATGAGGGTTTAGTTCGTTAAACATGAGAGCTTAGTACGTTAAACATGAGAGCTTAGTACG
TGAAACATGAGAGCTTAGTACGTACTATCAACAGGTTGAACTGCTGATCTTCAGATCCTCTACGCCGG
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AATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACAT
AAACAGTAATACAAGGGGTGTT*atgagccatattcaacgggaaacgtcttgctcgaggccgcgattaa*
attccaacatggatgctgatttatatgggtataaatgggctcgcgataatgtcgggcaatcaggtgcg
acaatctatcgattgtatgggaagcccgatgcgccagagttgtttctgaaacatggcaaaggtagcgt
tgccaatgatgttacagatgagatggtcagactaaactggctgacggaatttatgcctcttccgaccа
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CTTGACGGGACGGCGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGAAGGATCAGATCACGCATC
TTCCCGACAACGCAGACCGTTCCGTGGCAAAGCAAAAGTTCAAAATCACCAACTGGTCCACCTACAAC
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GTCAGCAACACCTTCTTCACGAGGCAGACCTCAGCGCCCCCCCCCCCCGAGCTCTTAATTAATTTAA
ATCTAGAGTCGACCTGCAGGCATGCAAGCTTCAGggttgagatgtgtataagagacagGCTCAACGTA
GCCTCAACCCGTATCATCCTGATGGATGGCCATACCGGTGCCGCAGCAGCTGGGCGGGTCGTTGAAGC
CTTTGGCCACTTCCTGGTCGGCGGCAACTTCGCCATCGGTATAGTGGTATTTATCATTTTGGTGGTGA

Figure 5.6: DNA sequence of the Tn-RL27 insert within the *flhA* gene of the non-motile serotype O1 (BA19/Tn-RL27) *Y. ruckeri* transconjugant. Regions highlighted in grey correspond to the *flhA* gene. Inverted repeats (underlined, lower case) are shown, as is the transposon-encoded aminoglycoside phosphotransferase (*aph*) gene (lower case, italics, underlined) and OriR6K replication site (italics). The sequencing primers tpnRL17-1 (italics, underlined) and tpnRL13-2 (underlined) are also included.

A

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CATTAACACTAACAGCCTGTCTTTGCTGACCAGACACCTGAACAAATCCCAGGGCTCTTTAGGCACCG
CCATTGAGCGTTTGTCTTCCGGTCTGCGCATCAACAGCGCTAAAGATGATGCAGCGGGTCAGGCGATT
GCCAACCGTttcacctctaacatcaacggCCTGACTCAGGCAGCACGTAACGCCAACGACGGTATTTC
ACTGTCTCAGACCGCTGAAGGCGCGTTGGGCGAAATCAACAACAACCTGCAACGTGTCCGTGACCTGA
CCGTGCAGGCGCAGAACAGCTCTAACTCTGCCTCTGATATCGATTCCATCCAGTCTGAAGTTAACCAG
CGTATGGAAGAAATCAACCGCGTCACCAAACAAACCGATTTCAACGGCATTAAAGTCTTGGATAACCG
CACGGCAGCCAACGCGGAATATGCTTTCCAGGTGGGTTCGCAAGATGCCCAGAAAATCAATATCGAAA
TTGGTTCAAGCGCGGGCTGGAACCTGGCGACTGCCGGTGCCGGTGGTACTTCATCTGATGTGGTGAAC
GATTCTACTCAGATTAGTAAAGCTAAAGAAACGGTAGTGCAAACGCTGTCAGGGAAAACTGAAGCACA
GATCAACACCGCACTGACCAAATTTACGACTGACGTTAAGGCGGCAACCGATGCTGCCGGTGTAGTCA
CGGCAAAAGGGGCGTTAACCACGGCTCTGGGTTTAAAAGCGGATGCCGATTTAGGTACCGCCGTCAGC
TCCGCTGCGTTTGGTACTGATTTAAGCGTCGACCAGATCGCTGGCGTGAAAAGTGGTGTTTACAGCGC
TGCAATTAACGGGGCAAATTACGCTACAGCAAAAACTGAAGCTGAAGTTAGCGCTGCTCAAGCGGGTG
CTAAAACTGCTGGTGCGATGGTTAACGGTAACTTCCGTTCCGTTGAAGCCAAAGGTTTTGACGTATTG
AAAGGTAACGTGACGggcggcgcaacgggtacagcAACGGGTACAACCCCACTGGCTGATATCGATGC
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TCACTAACCTGAACAACACCGTGAACAACTTGACTTCAGCCCGTAGCCGTATCCAAGATGCGGATTAC
TCTACCGAAGTATCCAACATGAGCCGTGCGCAGATCCTGCAACAAGCCGGGACTTCTGTTATGGCTCA
GGCTAACCAGGTTCCACAGACTGTCTTGTCTCTGCTGCGTCTCGAGCACCACCACCACCACCACTGA

B

MGSSHHHHHHSSGLVPRGSHMAVINTNSLSLLTRHLNKSQGSLGTAIERLSSGLRINSAKDDAAGQAI
ANRFTSNINGLTQAARNANDGISLSQTAEGALGEINNNLQRVRDLTVQAQNSSNSASDIDSIQSEVNQ
RMEEINRVTKQTDFNGIKVLDNRTAANAEYAFQVGSQDAQKINIEIGSSAGWNLATAGAGGTSSDVVN
DSTQISKAKETVVQTLSGKTEAQINTALTKFTTDVKAATDAAGVVTAKGALTTALGLKADADLGTAVS
SAAFGTDLSVDQIAGVKSGVYSAAINGANYATAKTEAEVSAAQAGAKTAGAMVNGNFRSVEAKGFDVL
KGNVTGGATGTATGTTPLADIDAALKAVDSQRSSLGASQNRFESTITNLNNTVNNLTSARSRIQDADY
STEVSNMSRAQILQQAGTSVMAQANQVPQTVLSLLRLEHHHHHH*

Figure 5.7: Open reading frame (ORF) of the cloned flagellin (*fliC*) gene from *Y. ruckeri* (**A**) and the deduced amino-acid sequence (**B**). Annealing sequences for the forward FLA-F (bold, underlined) and reverse FLA-R (underlined) primer are shown (**A**). In addition, annealing sites for sequencing primers FLG-L (lower case) and FLG-R (lower case, underlined) are also included. DNA sequences encoding the histidine (H) tags are highlighted (**A**), as are the predicted H-tags in the protein sequences (**B**).

A

ATGAAAAAGACAGCTATCACGCATAGCAGTGCACTGGCTGGTTTCGCTACAGTAGCGCAAGCCGCACC
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CAGACATTAGCAACGACGGTCCAACTCATAAGAGTCAACTGGGTGCTGGTGCATTCGTCGGTTACCAA
GCGAACCAGTATCTTGGCTTCGAAATGGGATATGACTGGCTGGGCCGTATGCCTTACAAAGGCGATAC
TGTTAACGGCGCTTTCaaagcacaaggcgttcagttGGCTGCTAAACTGAGCTACCCAATTGCTCAAG
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AGTAACCAAAAACTGGGCTACTCGTCTGGATTACCAATGGGTTAACAACATCGGTGACCGTGGTACCG
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ATCGTTCCAGTTGTTGCTCCAGCTCCAGCTCCAGCTCCAGTTGTTGATACCAAGCGTTTCACACTGAA
ATCTGACGTGCTGTTTGCTTTCAACAAAGCAACTCTGAAACCAGAAGGCCAG**caagcgctggatcaac**
tgtaTTCTCAGTTGAGCTCTATCGATCCTAAAGACGGTTCTGTTGTAGTTCTGGGCTTCGCTGACCGT
ATCGGTCAAGCTGCTCCTAACTTGAAACTGTCTGAAAACCGCGCTCGCAGCGTTGTAGAGTACCTGGT
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CAGTTACAGGTAACACCTGTGACAACGTGAAACCACGTGCTGCCCTGATCGAATGTCTGGCACCAGAT
CGTCGCGTAGAGATCGAAGTGAAAGGCATCAAAGAAGTTG**TGACTCAGCCACAGGCTTAA**

B

MKKTAITHSSALAGFATVAQAAPKDNTWYTGGKLGWSQFHDVGTGSDISNDGPTHKSQLGAGAFVGYQ
ANQYLGFEMGYDWLGRMPYKGDTVNGAFKAQGVQLAAKLSYPIAQDLDLYTRLGGMVWRADASVNEPS
TNSHASAHDTGVSPLAAVGLEYAVTKNWATRLDYQWVNNIGDRGTVGARPDNGMLSVGVSYRFGQDDA
IVPVVAPAPAPAPVVDTKRFTLKSDVLFAFNKATLKPEGQQALDQLYSQLSSIDPKDGSVVVLGFADR
IGQAAPNLKLSENRARSVVEYLVAKGIPADKISARGMGQADPVTGNTCW*

Figure 5.8: Sequence of the *Y. ruckeri ompA* gene (**A**) and the predicted protein sequence encoded by this ORF (**B**). Annealing sites for primers OMPA-F (underlined) and OMPA-R (bold, underlined) are shown. Sequencing primer annealing sites for OMPA-F1 (bold, lower case) and OMPA-R1 (lower case, underlined) are also included.

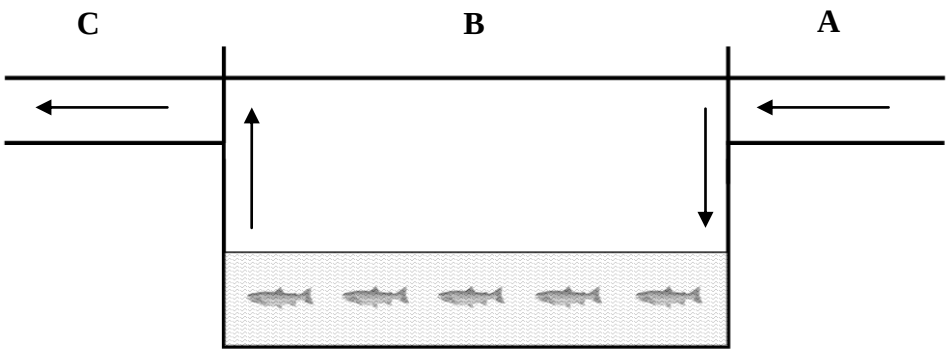

Figure 5.9: Diagrammatic representation of a prospective technique for administering recombinant flagellin to rainbow trout on a commercial scale. **A:** Naïve fish (average weight = 2 to 4 grams) are pumped from an early stage rearing tank into a vaccination chamber. **B:** The water level is reduced to a specific volume before the addition of a concentrated r-flagellin preparation. At this time, other bacterins and/or virus preparations could be applied. **C:** Water levels are increased before pumping vaccinates into a sterilized rearing tank.

A

MGSS**HHHHHH**SSGLVPRGSHMAVINTNSLSLLTRHLNKSQGSLGTAIERLSSGLRINSAKDDAAGQAI
ANRFTSNINGLTQAARNANDGISLSQTAEGALGEINNNLQRVRDLTVQAQNSSNSASDIDSIQSEVNQ
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DSTQISKAKETVVQTLSGKTEAQINTALTKFTTDVKAATDAAGVVTAKGALTTALGLKADADLGTAVS
SAAFGTDLSVDQIAGVKSGVYSAAINGANYATAKTEAEVSAAQAGAKTAGAMVNGNFRSVEAKGFDVL
KGNVTGGATGTATGTTPLADIDAALKAVDSQRSSLGASQNRFESTITNLNNTVNNLTSARSRIQDADY
STEVSNMSRAQILQQAGTSVMAQANQVPQTVLSLLRLE**HHHHHH***

B

HHHHHHSSGLVPRGSHMAVINTNSLSLLTRHLNKSQGSLGTAIERLSSGLRINSAKDDAAGQAIANRF
TSNINGLTQAARNANDGISLSQTAEGALGEINNNLQRVRDLTVQAQNSSNSASDIDSIQSEVNQRMEE
INRVTKQTDFNGIKVLDNRTAANAEYAFQVGSQDAQKINIEIGSSAGWNLATAGAGGTSS**HHHHHH***

C

HHHHHHVNGNFRSVEAKGFDVLKGNVTGGATGTATGTTPLADIDAALKAVDSQRSSLGASQNRFESTI
TNLNNTVNNLTSARSRIQDADYSTEVSNMSRAQILQQAGTSVMAQANQVPQTVLSLLRLE**HHHHHH***

D

HHHHHHSSGLVPRGSHMAVINTNSLSLLTRHLNKSQGSLGTAIERLSSGLRINSAKDDAAGQAIANRF
TSNINGLTQAARNANDGISLSQTAEGALGEINNNLQRVRDLTVQAQNSSNSASDIDSIQSEVNQRMEE
INRVTKQTDFNGIKVLDNRTAANAEYAFQVGSQDAQKINIEIGSSAGWNLATAGAGGTSSSSSSVNGN
FRSVEAKGFDVLKGNVTGGATGTATGTTPLADIDAALKAVDSQRSSLGASQNRFESTITNLNNTVNNL
TSARSRIQDADYSTEVSNMSRAQILQQAGTSVMAQANQVPQTVLSLLRLE**HHHHHH***

Figure 5.10: Potential proteins/peptides derived from the recombinant *Y. ruckeri* flagellin protein sequence which could be used to refine the r-flagellin vaccine. **A:** Full amino-acid sequence of the *Y. ruckeri* r-flagellin protein used as template. **B:** N-terminal amino-acid sequence which is highly conserved between the *Y. ruckeri* flagellin protein and that of other Enterobacteriaceae flagellins. **C:** C-terminal amino-acid sequence which is similarly conserved among different flagellin proteins. **D:** Conserved N- and C- terminal fusion protein linked by a serine linker peptide (S). All proteins contain the Histidine tag (**H**) at the N- and C- terminal as they would be expressed using pET28-b vector and purified by passing through an IMAC column.